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ORIGINAL ARTICLE

In vitro cytotoxic effect of tyrosine kinase inhibitor sunitinib malate alone and in combination with hyperthermia on breast adenocarcinoma MCF-7 cells

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Summary

Purpose: *In this study, the in vitro cytotoxic effect of sunitinib malate alone and combination with hyperthermia was evaluated on MCF-7 cells (human breast adenocarcinoma cell line).*

Methods: *For this purpose cell proliferation assay, mitotic index and labelling index analysis among cell kinetic parameters were assessed. Sunitinib malate doses of 1, 5 and 10 μM were applied alone and in combination with hyperthermia to cells for 24-72 hrs.*

Results: *A significant decrease ($p<0.05$) was noticed in cell proliferation, mitotic index and labelling index for all experimental groups and for all applications.*

Conclusion: *Labeling index and mitotic index values show that sunitinib malate combined with hyperthermia was significantly more effective in MCF-7 cells than when given alone. This combination acts through synergistic and additive effects.*

Key words: *breast cancer, cell kinetic, hyperthermia, MCF-7 cells, sunitinib malate*

Introduction

Breast cancer is the most frequent cancer type among women worldwide [1-3]. The frequent failures of conventional therapies have led researchers to attempt to find new treatment methods. Hyperthermia effects angiogenesis and cancer stem cells. It is suggested that application of hyperthermia combined with chemotherapy, radiotherapy and gene therapy can improve cancer therapy [4]. Hyperthermia is a treatment method based on raising the temperature of the entire body or local body areas to 41-43 °C; as a result cancer cells become more sensitive to other treatment methods such as chemotherapy and radiotherapy [5]. It is in fact ex-method [6,7] nevertheless this method is currently not very well known. On the other hand it has been shown that hyperthermia combined with radiotherapy and/or chemotherapy produces beneficial results in patients [8] and can be used in the clinic [9].

Tyrosine kinase receptors can be targeted either at the extracellular or the intracellular level with monoclonal antibodies and small molecules, respectively [10].

Sunitinib malate is a multitargeted inhibitor of tyrosine kinase receptors, such as platelet-derived growth factor receptors (PDGFRα and PDGFRβ), vascular endothelial growth factor receptors 1-3 (VEGFR1–VEGFR3), stem cell factor receptor (Kit), Flt-3, and colony-stimulating factor-1 receptor (CSF-1R) [11,12].

The aim of the present study was to investigate the effect of sunitinib malate alone and in combination with hyperthermia on MCF-7 cell line using cell kinetic parameters including cell proliferation assay, mitotic index and labelling index.

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Methods

The MCF-7 cell line used in this study was obtained from European Cell Culture Collection. Cells were cultured in RPMI 1640 (Gibco Lab, Scotland, UK) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 µg/ml streptomycin (streptomycin sulphate, I.E. Ulugay, Istanbul, Turkey), 100 IU/ml penicilin (Pronapen, Pfizer, Istanbul, Turkey), amphotericin B (Sigma, St.Louis, USA) and 2 mM glutamine at 37°C in humidified atmosphere of 5% CO_2. The pH of the medium was adjusted to 7.4 with $NaHCO_3$.

Sunitinib malate concentrations that were used in the present study were determined based on previous *in vitro* and clinical studies [13-16]. First, 1 mM stock solution was prepared with RPMI-1640 supplemented with 10% FBS. Three different doses were obtained by dilution of the stock solution and were determined as dose 1 (D1) = 1 µM, dose 2 (D2) = 5 µM and dose 3 (D3) = 10 µM. MCF-7 cell cultures were exposed to the 3 different doses alone and in combination with hyperthermia for 24, 48 and 72 hrs.

In order to mimic a conventional whole body hyperthermia, a cell culture incubator was used as the energy source. The temperature of the incubator was set at 43°C. Twenty-four hrs after cell seeding plates that were originally incubated in the 37°C incubator were transferred to the 43°C incubator for 1 hr.

$2x10^4$ cells were seeded in the 96-well plate with 200 µl per well for each experimental group. At the end of the experimental period the medium in each well was removed and 40 µl fresh MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma, Missouri, USA) solution (5 mg/ml in phosphate buffered saline/PBS, Sigma, St.Louis, USA) was added into each well and cells were incubated at 37°C for 4 hrs. Then, 16 µl dimethylsulphoxide (DMSO) (Sigma) were added into each well and cells were shaked thoroughly for 1 hr on a shaker. The absorbance of the samples was measured against a background control as a blank using an Elisa reader (µQuant, Bio-Tek Instruments Inc, Vermont, USA) at 450-690 nm.

The mitotic index was studied using the Feulgen method [17,18]. For mitotic index analysis MCF-7 cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well. Before the cells were stained with Feulgen, they were prepared with 1 N HCl at room temperature for 1 min and then hydrolized with 1 N HCl for 10.5 min at 60°C. After slides were stained with Feulgen, they were rinsed for a few minutes in distilled water and stained with 10 % Giemsa stain solution at pH 6.8 for 3 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water and then were air-dried. Finally, the mitotic index was calculated by counting metaphases, anaphases and telophases for each control and the experimental groups. At least 3,000 cells were examined using light microscope from each slide to determine the mitotic index.

For ^{3}H-thymidine labelling index analysis which determines cells in the S phase, MCF-7 cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well and incubated overnight. Then, the cells were treated with 1, 5 and 10 µM sunitinib malate concentrations alone and in combination with hyperthermia. At the end of the experimental period, cells were treated with medium containing 1 µCi/mL 3H-thymidine for 20 min to evaluate the labelling index.

After labelling, the cells were fixed with Carnoy's fixative and the remaining radioactive material was washed twice with 2% perchloric acid at 4°C for 30 min.

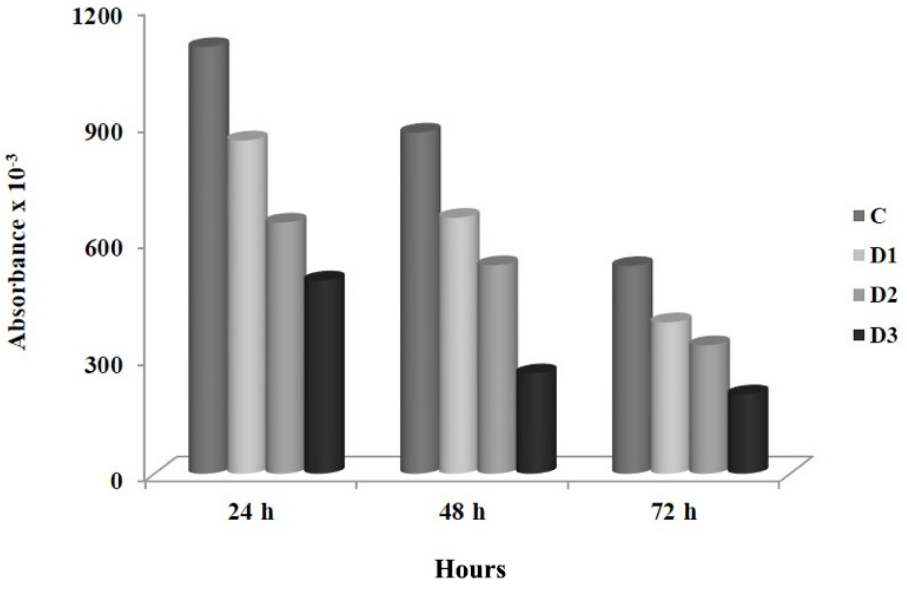

Figure 1. Absorbance values of mitochondrial dehydrogenase activity of MCF-7 cells treated with D1, D2 and D3 dose of sunitinib malate (D1:1 µM, D2:5 µM and D3:10 µM) for 0-72 hrs (450-690 nm) ($p<0.05$). C: untreated cells.

Coverslips were prepared and were coated with K.2 gel emulsion (Ilford, Cheshire, UK) at 40°C to determine thymidine labelling index. After 3 days exposure at 4 °C, autoradiograms were washed with D-19 b developer (Kodak, New York, USA) and fixed with Fixaj B (Kodak). The coverslips were evaluated after being stained with Giemsa for 3 min. The labelled cells were counted on each coverslip. At least 3,000 cells were examined from each coverslip.

Statistics

The mitotic index and the apoptotic index were investigated relative to controls and to each other for evaluation of the changes in proliferation. Data obtained from all experimental groups were analyzed using one-way ANOVA test. The statistically significance between control and experimental groups was determined by Dunnett's test and the significance between experimental groups was determined by Student's t-test. A p value <0.05 was considered as statistically significant.

Results

After administration of 1, 5 and 10 μM sunitinib malate alone and in combination with hyperthermia for 0-72 hrs, the viability values of MCF-7 cells decreased significantly. The differences between the control and all experimental groups were significant ($p<0.05$). As seen in Figure 1 cell viability values decreased from $1097x10^{-3}$ to $857x10^{-3}$, $646x10^{-3}$ and $497x10^{-3}$ at 24 hrs; from $877x10^{-3}$ to $658 x10^{-3}$, $536x10^{-3}$ and $258x10^{-3}$ at 48 hrs; and from $534x10^{-3}$ to $389x10^{-3}$, $330x10^{-3}$ and $204x10^{-3}$ at 72 hrs, respectively for 1, 5 and 10 μM sunitinib malate concentrations alone. In Figure 2 cell viability values decreased from 1001x10-3 to $825x10^{-3}$, $619x10^{-3}$ and $484x10^{-3}$ at 24 hrs; from $675x10^{-3}$ to $445x10^{-3}$, $325x10^{-3}$ and $219x10^{-3}$ at 48 hrs; and from $511x10^{-3}$ to $346x10^{-3}$, $294x10^{-3}$ and $86x10^{-3}$ at 72 hrs for 1, 5 and 10 μM sunitinib malate concentrations in combination with hyperthermia. Cell viability values caused by sunitinib malate alone and in combination with hyperthermia decreased in a time-dependent manner.

After administration of 1, 5 and 10 μM sunitinib malate concentrations alone and in combination with hyperthermia for 0-72 hrs, the mitotic index values of MCF-7 cells decreased significantly. The differences between the control and all experimental groups were significant ($p<0.05$). As seen in Figure 3 the mitotic index values decreased from 3.03 to 2.89, 2.04, and 1.65 at 24 hrs; from 3.69 to 2.97, 1.78, and 1.19 at 48 hrs; and from 4.49 to 2.61, 1.26, and 0.41 at 72 hrs, respectively for 1, 5 and 10 μM sunitinib malate concentrations alone. Figure 4 shows that the mitotic index values decreased from 2.89 to 2.62, 1.48, and 0.96 at 24 hrs; from 3.37 to 2.86, 1.67, and 0.62 at 48 hrs; and from 3.94 to 2.18, 1.04, and 0.17 at 72 hrs, respectively for 1, 5 and 10 μM sunitinib malate concentrations in combination with hyperthermia. The mitotic index values caused by sunitinib malate alone and in combination with hyperthermia decreased in a time-dependent manner.

After administration of 1, 5 and 10 μM su-

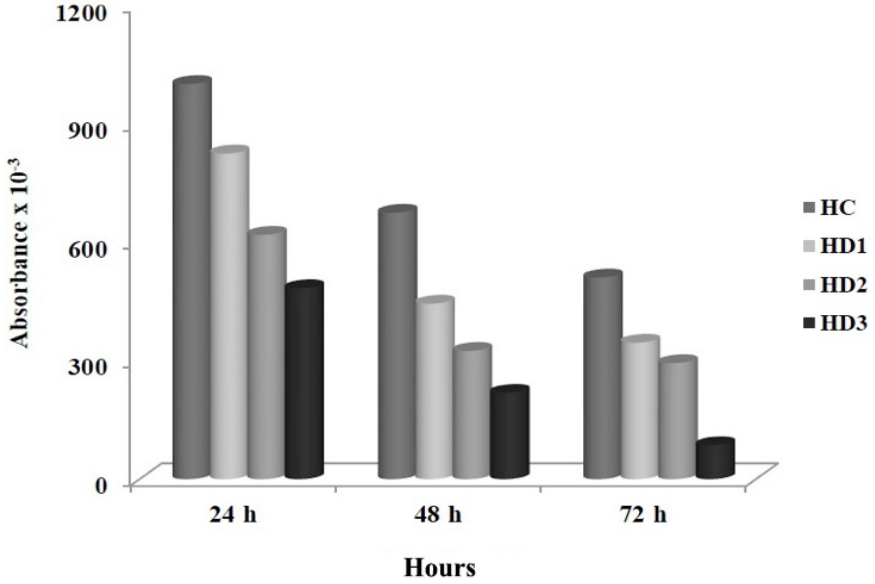

Figure 2. Absorbance values of mitochondrial dehydrogenase activity of MCF-7 cells treated with HD1, HD2 and HD3 dose of sunitinib malate combined with hyperthermia (HD1: 1 μM, HD2: 5 μM and HD3: 10 μM) for 0-72 hrs (450-690 nm) ($p<0.05$). HC: cells treated with hyperthermia alone. HD: hyperthermia+sunitinib dose.

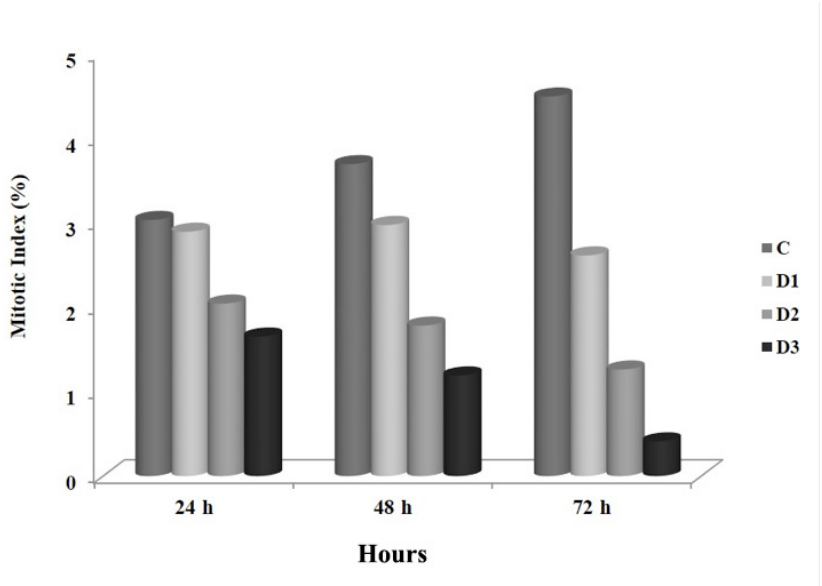

Figure 3. Mitotic index (%) values of MCF-7 cells treated with D1, D2 and D3 dose of sunitinib malate (D1:1 μM, D2:5 μM and D3:10 μM) for 0-72 hrs ($p<0.05$). C: untreated cells.

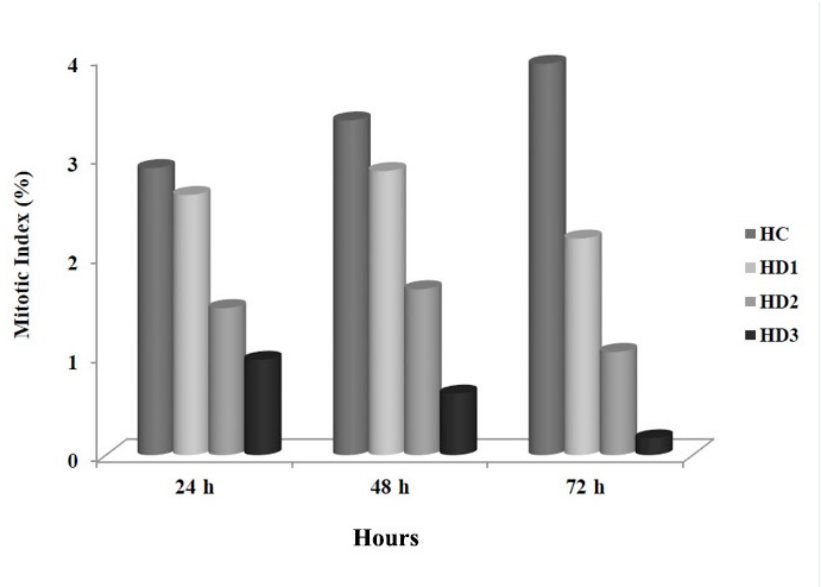

Figure 4. Mitotic index (%) values of MCF-7 cells treated with HD1, HD2 and HD3 dose of sunitinib malate combined with hyperthermia (HD1:1 μM, HD2:5 μM and HD3:10 μM) for 0-72 hrs ($p<0.05$). HC: cells treated with hyperthermia alone. HD: hyperthermia+sunitinib dose.

nitinib malate alone and in combination with hyperthermia for 0-72 hrs, the labelling index values of MCF-7 cells decreased significantly. The differences between the control and all experimental groups were significant ($p<0.05$).

As seen in Figure 5, the labelling index values decreased from 5.5 to 4.7, 3.9, and 3.2 at 24 hrs; from 6 to 4, 3.3 and 3 at 48 hrs; and from 6.6 to 3.5, 3.1, and 2.2 at 72 hrs respectively for 1, 5 and 10 μM sunitinib malate concentrations alone. As shown in Figure 6, the labelling index values decreased from 4 to 3.2, 2.8, and 2.6 at 24 hrs; from 5 to 3.2, 3, and 2.5 at 48 hrs; and from 5.2 to 2.8, 2.1, and 1.1 at 72 hrs, respectively for 1, 5 and 10 μM sunitinib malate concentrations in combination with hyperthermia. The labelling index values caused by sunitinib malate alone and in combination with hyperthermia decreased in a time-dependent manner.

Discussion

The goal of this study was to evaluate and compare the cytotoxic effects of sunitinib malate alone and in combination with hyperthermia on MCF-7 cell line which originates from estrogen

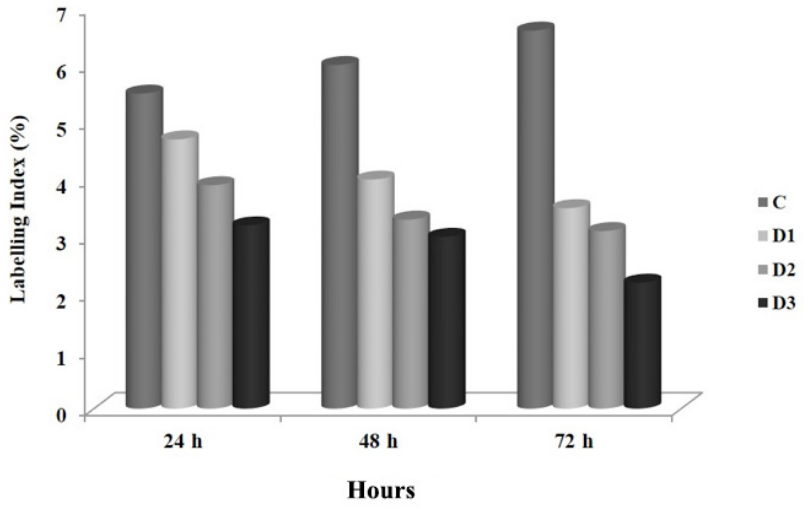

Figure 5. Labelling index (%) values of MCF-7 cells treated with D1, D2 and D3 dose of sunitinib malate (D1:1 μM, D2:5 μM and D3:10 μM) for 0-72 hrs ($p<0.05$). C: untreated cells.

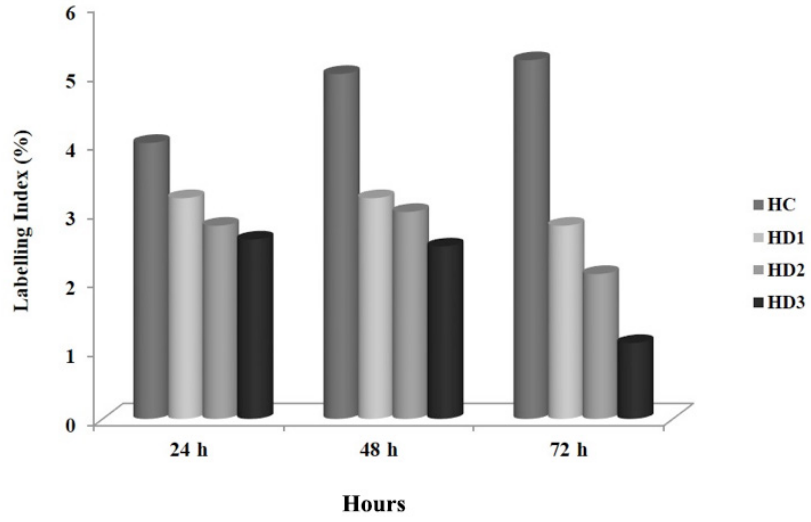

Figure 6. Labelling index (%) values of MCF-7 cells treated with HD1, HD2 and HD3 dose of sunitinib malate combined with hyperthermia (HD1:1 μM, HD2:5 μM and HD3:10 μM) for 0-72 hrs ($p<0.05$). HC: cells treated with hyperthermia alone. HD: hyperthermia+sunitinib dose.

receptor positive human breast adenocarcinoma. For this purpose various cell kinetic parameters including cell proliferation assay, mitotic index and labelling index analysis were used.

Although cytotoxic chemotherapy has an important place in cancer treatment, it poses various limitations. Targeting not only cancer cells but also healthy cells creates various adverse effects that reduce the quality of patients' life. Besides, most of the conventional chemotherapy administered to patients remains palliative rather than therapeutic. To improve the quality of patients' life and, of course, the effectiveness of the treatment, searching for new drugs is on the increase. In this context, developed targeted drugs constitute a great hope for patients [19].

Sunitinib malate has shown antiangiogenic and antitumor activities in several in vitro and in vivo tumor models [20-25]. In preclinical models, sunitinib malate administration has shown a significant reduction of phosphotyrosine levels of VEGFR-2, PDGFR-® and KIT, which correlated

with tumor growth inhibition [14,15]. In a study by Tekisogullari and Topcul, it was shown that there were an increase in the apoptotic index and a decrease in the mitotic index and cell proliferation rate after administration of 10 μM sunitinib dose to HeLa cells, especially at 72 hrs [27].

Hyperthermia can affect cells in S phase, inhibit sublethal damage repair and improve oxygenation. All these make hyperthermia an attractive therapy combination with radiotherapy and/or chemotherapy [28-32]. It can be used to treat cancer in many parts of the body such as brain, thyroid, lung, breast, colon, stomach, ovary, and prostate [33-41]. Radiotherapy and chemotherapy were inefficient in patients with local metastases but application of these treatment methods combined with hyperthermia led to tumor shrinkage [42]. Hyperthermia can modulate the action of various anticancer drugs and contribute to DNA damage arising from temperature [43]. Depending on preclinical research, the effects of hyperthermia are mainly due to enhancement of drug efficacy or thermal radiosensitization, and to a lower extent to direct cytotoxicity within areas of tumors heated up to more than 42.5 °C [44,45]. In a study by Trieb et al. decrease in proliferation was measured with MTT test. One hr after administration of 10 μg/ml paclitaxel and 5 μg/ml carboplatin in combination with hyperthermia at 43°C, significantly decreased cell proliferation was noticed compared with administration of 10 μg/ml paclitaxel and 5 μg/ml carboplatin alone ($p<0.01$) [34]. Tang et al. indicated that the combination of curcumin with hyperthermia significantly inhibited cell proliferation of murine Lewis lung carcinoma cell line LL/2 and endothelial cell line MS1 *in vitro* [46].

In the present study, antiproliferative effects of especially 10 μM sunitinib malate alone on MCF-7 were seen. Besides, the results of our study suggest that sunitinib malate in combination with hyperthermia could further improve the effectiveness of treatment of luminal A type MCF-7 breast cancer cell line. Consequently, the data obtained from sunitinib malate in combination with hyperthermia suggests that hyperthermia can also be used in clinical practice to increase the effectiveness of the drug.

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Conflict of interests

The authors declare no confict of interests.

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