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Chemotherapeutic Effect of Stigmasterol in Sorafenib Treated Breast Cancer Cell Lines *via* Modulation of NF-κB and ERK Signaling Pathways

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Abstract

Breast cancer is the main predisposing factor for female tumor-related death globally, which increases the requirement to investigate the effectiveness of new drug combination strategies. Sorafenib is a multi-kinase inhibitor mainly targeting vascular endothelial growth factor receptor (VEGFR) and Ras/Raf/ Extracellular signal-regulated kinase (ERK) pathway. Stigmasterol is a phytosterol with anticancer activity targeting different oncogenic pathways. This study aimed to examine the antitumor effects of stigmasterol and sorafenib combination against MDA-MB-231 and MCF-7 breast cancer cell lines via assessing their impact on VEGF, VEGFR-2, nuclear factor kappa B (NF-κB), Ki-67, Bcl-2, ERK, and caspase-3. Cytotoxicity was investigated using the MTT assay. VEGF, VEGFR-2, ERK, NF-κB, Bcl-2, and Ki-67 levels were assessed using the ELISA technique. VEGFR-2 gene expression was assessed using the RT-PCR technique, while caspase-3 activity was investigated using the colorimetric technique. Sorafenib and stigmasterol combination reduced the levels of NF-κB, Bcl-2, Ki-67, VEGFR-2, and VEGF-A, whereas the activity of caspase-3 was increased. Stigmasterol and sorafenib combination may be a promising therapeutic regimen for breast cancer treatment through modulation of NF-κB-VEGF/BCL-2 and ERK/Caspase-3 signaling axes crosstalk.

Keywords: Breast cancer, sorafenib, stigmasterol, angiogenesis, apoptosis.

1. Introduction

Breast cancer represents the most prevalent female-diagnosed cancer and causes the highest fatality rate in women worldwide [1]. Breast cancer progression is significantly affected by sustained proliferation, excessive angiogenesis, and apoptosis evasion [2].

Targeting vascular endothelial growth factor (VEGF) represents valuable cancer therapy [3]. VEGF modulates angiogenesis [4]. It also promotes the up-regulation of Bcl-2, leading to apoptosis inhibition [5]. Moreover, VEGF has been identified as a target of nuclear factor kappa B (NF-κB) inhibition [6, 7]. The NF-κB signaling pathway controls proliferation, cell cycle, apoptosis, inflammation, and invasion through gene expression regulation [8]. On the other hand, VEGF signaling activates NF-κB [4].

Sorafenib is an oral multi-kinase inhibitor that inhibits the signaling of vascular endothelial growth factor receptor [9]. It also inhibits the phosphorylation of downstream RAF/MEK/ extracellular signal-regulated kinase (ERK) signaling [9].

ERK signaling is essential in almost all cellular processes, and therefore its activity must be precisely controlled [10]. Various anti-proliferative functions could be mediated by ERK activation depending on cell stimulus and type [11].

Activation of ERK induces apoptotic pathways. This effect requires sustained ERK activity in specific subcellular compartments [11].

FDA has approved sorafenib for advanced hepatocellular carcinoma (HCC) treatment [9]. However, ERK signaling reactivation induced acquired resistance and diminished sorafenib therapeutic benefits in sorafenib-resistant HCC cells [12].

Many studies are ongoing to develop complementary or alternative sorafenib therapy

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regimens to overcome its acquired resistance [13]. Multiple clinical trials have shown that the combination between sorafenib and adjuvant products is promising for breast cancer treatment. However, the results are still not approved for breast cancer treatment [14, 15].

Several natural compounds exhibit antitumor activity and can be used for cancer therapy [16]. These agents act via several mechanisms, including apoptosis induction which is gaining interest as an alternative approach to target cancers [17]. Furthermore, phytochemicals are beneficial to counteract drug resistance and alleviate the side effects of chemotherapy [16].

Stigmasterol is a type of phytosterol. It is frequently found in vegetables and fruits [18]. Several stigmasterol antitumor effects were previously detected. ERK signaling was suppressed by stigmasterol in ovarian cancer [19]. The pro-apoptotic impact was also observed in ovarian [19], gastric [20, 21], hepatic [22], and gall bladder [23] cancers. In addition, VEGFR-2 gene expression was down-regulated in cholangiocarcinoma [24].

Although several phytochemicals showed the ability to augment sorafenib-antitumor efficacy [25], the effects of a stigmasterol and sorafenib combination have not been studied yet.

On this basis, the purpose of the current study was to examine the antitumor effects exerted by the combination of stigmasterol and sorafenib against MDA-MB-231 and MCF-7 breast cancer cell lines via assessing their impact on apoptosis, angiogenesis, and proliferation via elucidating NFκB-VEGF/BCL-2 and ERK/caspase-3 pathways crosstalk.

2. Material and methods

2.1. Drugs

Stigmasterol (~95%) and sorafenib tosylate (≥98 % HPLC) were purchased from Sigma-Aldrich (St. Louis, USA). 10 mM sorafenib and 100 mM stigmasterol stock solutions were prepared by dissolving them in DMSO. Then, until usage, stock solutions were stored at refrigerator temperature.

2.2. Chemicals

Dimethyl Sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), phosphate buffered saline, penicillin-streptomycin (pen-strep), trypsin, Dimethyl Sulfoxide (DMSO), and MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltrazolium bromide) were obtained from Maadi Medical Supplies (Cairo, Egypt). Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (St. Louis, USA).

2.3. Cell lines

MDA-MB-231 and MCF-7 were different human breast cancer cell lines. They were gained from American Type Culture Collection (ATCC, USA). Both types of cells represent an example of invasive breast cancer, but they have several differences: whereas MCF-7 is estrogen and progesterone dependent, MDA-MB-231 is triple-negative [26].

2.4. Cell cultures

T-75 tissue culture flasks were used to culture monolayers of cells. DMEM was utilized as a culture medium containing 1% penicillin-streptomycin and 10% FBS. Incubation proceeded at 37°C in a humidified incubator containing 5% CO2. After reaching 80–90% confluence, cells were passaged.

2.5. Assessment of cell viability

MTT assay was performed for cell viability investigation [27]. Initially, cell seeding was conducted in 96-well plates, and each well contained 200 µL of complete medium. After overnight incubation at 37°C in 5% CO2, the old medium was replaced with a new one containing various concentrations of drugs. The examined concentrations for stigmasterol were 35 µM, 70 µM, 140 µM, 280 µM, and 560 µM, while sorafenib concentrations were 3 µM, 6 µM, 12 µM, 24 µM, and 48 µM. Plates were then incubated for 48 hours, followed by the elimination of the medium and addition of 50 µL MTT solution (5mg/mL in phosphate-buffered saline). MTT solution was removed after incubation for 4 hours. The crystals were dissolved by addition of 50 µL DMSO in each well. The absorbance was measured at a wavelength of 490 nm using a microplate reader then the percentages of drug-treated wells/ untreated control wells were calculated to represent cell viability.

2.6. Experimental design

Ethical Committee of the Faculty of Pharmacy, Damanhour University, approved the conduction of this study (Ref.no 1219PB14). Both cell types were cultured to make four experimental groups. Three replicate flasks were cultured for

each group. The first group was treated with sorafenib (21.5 µM for MDA-MB-231 and 18.6 µM for MCF-7). Both cell lines in the second group were treated with 1,000 µM stigmasterol, and the third group was treated with stigmasterol and sorafenib combination. The control group was the fourth one. Cells were harvested after 48 hours of incubation.

2.7. Cell lysate preparation and BCA assay

A cell scraper was initially utilized for cell elimination from the surface of the flasks. Following the centrifugation of cell suspensions at 600xg for 5min, the supernatants were removed. Phosphate-buffered saline was then used for cell re-suspension, followed by centrifugation and supernatant removal. Afterwards, cell lysate was prepared by addition of RIPA Lysis Buffer (Catalog Number: AR0105, Boster Biological Technology, Pleasanton, USA) to cell pellets. Following 30 minutes of incubation on ice, centrifugation was made for 10 minutes at 14000xg. Finally, new tubes were used to store cell lysates (supernatants) until analysis [28].

Pierce™ BCA Protein Assay Kit (Catalog number: 23225, Thermo fisher scientific, USA) was employed dependent on the manufacturer's instructions for quantification of total protein.

2.8. Biochemical analyses

2.8.1. Markers of proliferation

Human Ki67 Simple Step ELISA® Kit (ab253221, Abcam, UK) was used following the manufacturer's instructions to analyze Ki-67 levels.

2.8.2. Markers of angiogenesis

2.8.2.1. VEGF and VEGFR-2 levels

Following the manufacturer's instructions, Human VEGF Receptor 2 Simple Step ELISA® Kit (ab213476, Abcam, UK) was used to detect the levels of VEGFR-2. The levels of VEGF were investigated using Human VEGF Quantikine ELISA Kit (Catalog Number: DVE00, Bio-Techne, USA).

2.8.2.2. Gene expression of VEGFR-2

In order to examine the expression of VEGFR-2 gene, the technique of one-step real-time reverse transcription polymerase chain reaction (RT-PCR) was conducted. First, miRNeasy Mini Kit (Cat. No: 217004, Qiagen, USA) was utilized for the extraction of total RNA following the kit instructions. Second, Rotor-Gene SYBR Green RT-PCR Kit (Cat. No: 204174, Qiagen, USA) was employed to conduct PCR reactions following the manufacturer's instructions.

5'CCTGGAGAATCAGACGACAA3' was the forward primer sequence of VEGFR-2, while the reverse primer sequence was 5'CCGGTTCCCATCCTTCAATA3'. The used housekeeping gene was (β Actin) whose primers sequence were F: 5'ACCATGGATGATGATATCGC3' and R: 5'CATAGGAATCCTTCTGACCCA3'.

The instrument was set at 55°C during reverse transcription for 10 min. The temperature was then changed to 95°C during PCR initial activation step for 5 min. Denaturation was performed at 95°C for 5 s followed by 10 s of annealing/extension at 60°C. PCR cycle was repeated for 35 times, and the results were compared using relative quantification (RQ) [29].

2.8.3. Markers of Apoptosis

Analyses of Bcl-2 levels and caspase-3 activity were performed, depending on the manufacturer's instructions, using Bcl-2 Human ELISA Kit ((ab119506), Abcam, UK), and Caspase-3 Colorimetric Assay Kit (Sigma Aldrich, USA) (Product Code CASP-3-C), respectively.

2.8.4. Other signaling markers

According to the kit instructions, the levels of p-ERK were measured using Human pERK1/2 ELISA Kit (Catalog No: MBS2511875, MyBioSource, USA). Human Nuclear Factor Kappa B (NF-κB) ELISA Kit (Catalog No: MBS450580, MyBioSource, USA) was utilized to analyze NF-κB levels.

2.9. Statistical analysis

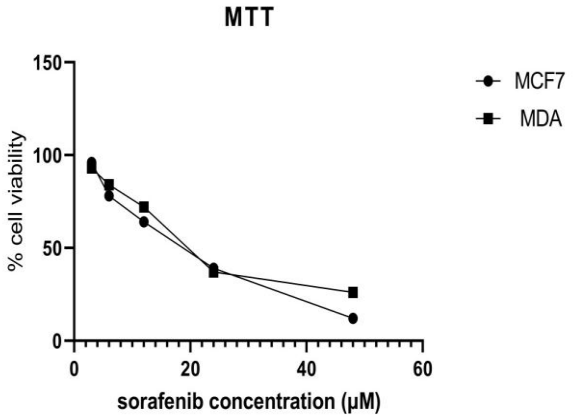
The mean ± standard deviation of the mean (SD) was the way to express the results of each experimental group then multiple comparisons were performed by version 8.0.2 of Graph Pad Prism Software. The software was set to carry out one-way analysis of variance test, which was then followed by the test of Tukey post hoc. The differences between the results were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. MTT assay

The growth of both MDA-MB-231 and MCF-7 cells was inhibited by sorafenib. The IC50 of sorafenib was 21.5 µM for MDA-MB-231 and 18.6 µM for MCF-7 (Fig.1). However, IC50 could

not be estimated for stigmasterol because of weak cytotoxicity in both cell types. The highest concentration of stigmasterol produced 70% cell viability, and subsequent work was completed



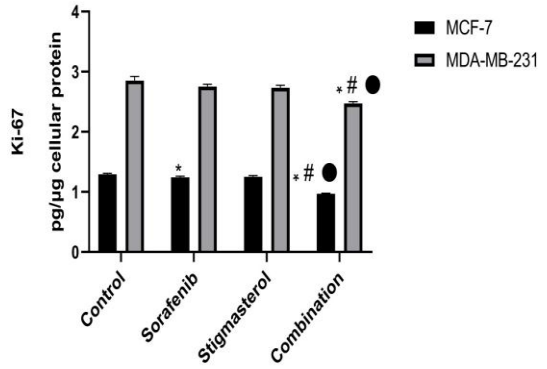
using one thousand µM of stigmasterol.

Fig. 1. *Effects of sorafenib on cell viability of MCF-7 and MDA-MB-231 analyzed by MTT assay. Data were presented as the means ± standard deviation of mean. Each concentration was performed in triplicate.*

3.2. Biochemical analyses

3.2.1. Impact of stigmasterol and sorafenib on cell proliferation

In MDA-MB-231 and MCF-7, a significant decrease of ki-67 levels cells was observed in stigmasterol and sorafenib combination group compared to either stigmasterol or sorafenib (p



≤0.05) (Fig.2).

Fig. 2. *Effects of sorafenib, stigmasterol and their combination on cell proliferation after 48 hours incubation with MCF-7 and MDA-MB-231. Ki67 was analyzed by ELISA technique and data were presented as the means ± standard deviation of mean of three samples each performed in triplicate. Statistically significant differences between groups were designated as *p < 0.05 vs. control, #p < 0.05 vs. sorafenib group and ●p< 0.05 vs. stigmasterol group.*

3.2.2. Impact of stigmasterol and sorafenib on angiogenesis

The combination of stigmasterol and sorafenib significantly suppressed VEGFR-2 and VEGF levels compared to either stigmasterol or sorafenib in MDA-MB-231 cells and MCF-7 (p≤ 0.05) (Fig.3).

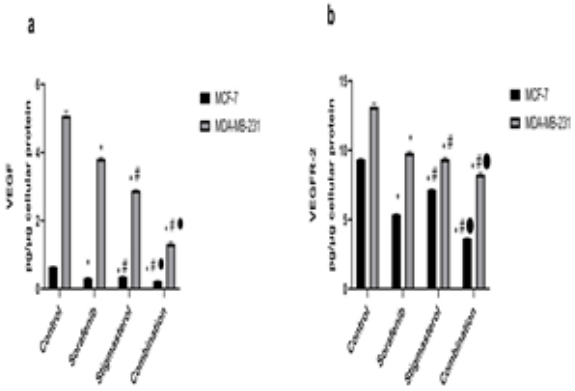


Fig. 3. *Effects of sorafenib, stigmasterol and their combination on angiogenesis after 48 hours incubation with MCF-7 and MDA-MB-231 cells. (a) VEGF, and (b) VEGFR-2 levels were analyzed by ELISA technique and data were presented as the means ± standard deviation of mean of three samples each performed in triplicate. Statistically significant differences between groups were designated as *p < 0.05 vs. control, #p < 0.05 vs. sorafenib group and ●p < 0.05 vs. stigmasterol group.*

A significant down-regulation of VEGFR-2 mRNA expression was also observed in stigmasterol and sorafenib combination compared to either sorafenib or stigmasterol in MCF7 and MDA-MB-231 cells (p≤ 0.05) (Fig.4).

3.2.3. Impact of stigmasterol and sorafenib and on apoptosis

The levels of Bcl-2 were significantly suppressed, while the activity of caspase-3 was significantly increased by stigmasterol and sorafenib combination compared to either sorafenib or stigmasterol in MDA-MB-231 and MCF-7 cells (p≤ 0.05) (Fig.5).

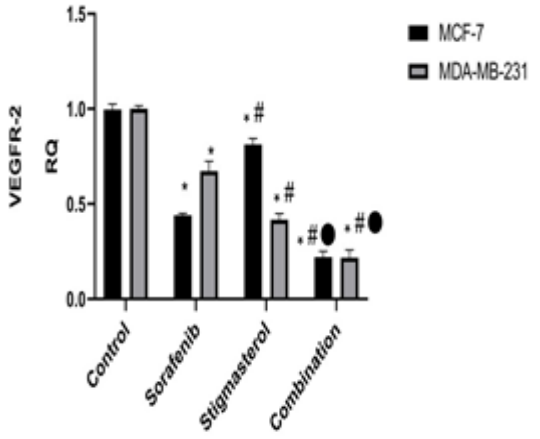


Fig. 4. *Effects of sorafenib, stigmasterol and their combination after 48 hours incubation on the VEGFR-2 m-RNA expression in MCF-7 and MDA-MB-231 cells. Results were measured by real-time PCR technique and data were presented as the means ± standard deviation of mean of three samples each performed in triplicate. Statistically significant differences between groups were designated as *p < 0.05 vs. control, #p < 0.05 vs. sorafenib group and ●p < 0.05 vs. stigmasterol group (RQ: relative quantification).*

3.2.4. Impact of stigmasterol and sorafenib on NF-κB and p-ERK levels

A significant low level of NF-κB was detected in the sorafenib and stigmasterol combination group compared to either sorafenib or stigmasterol in MDA-MB-231 and MCF-7 cells ($p \leq 0.05$) (Fig.6).

P-ERK level in the sorafenib and stigmasterol combination group was non-significant compared to either sorafenib or stigmasterol in MCF-7 and MDA-MB-231 cells. ($p \leq 0.05$) (Fig.6).

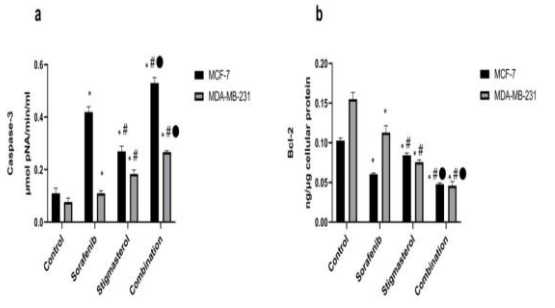


Fig. 5. Effects of sorafenib, stigmasterol and their combination on apoptosis after 48 hours incubation with MCF-7 and MDA-MB-231 cells. (a) Caspase-3 activity and (b) Bcl-2 level were analyzed by colorimetric and ELISA techniques respectively and data were presented as the means ± standard deviation of mean of three samples each performed in triplicate. Statistically significant differences between groups were designated as *p < 0.05 vs. control, #p < 0.05 vs. sorafenib group and ●p < 0.05 vs stigmasterol group.

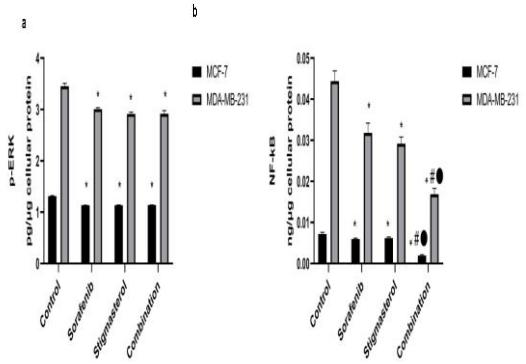


Fig. 6*. Effects of sorafenib, stigmasterol and their combination on (a) P-ERK, and (b) NF-κB levels after 48 hours incubation with MCF-7 and MDA-MB-231 cells. The levels were analyzed by ELISA technique and data were presented as the means ± standard deviation of mean of three samples each performed in triplicate. Statistically significant differences between groups were designated as *p < 0.05 vs. control, #p < 0.05 vs. sorafenib group and ●p < 0.05 vs stigmasterol group.*

4. Discussion

Breast cancer represents a serious worldwide health concern and is responsible for the majority of female cancer-associated deaths [1]. In addition, highly varied breast cancer subtypes might become resistant to conventional treatment, which results in a persistent demand for novel therapeutic approaches [30].

The present study aimed to explore the antitumor effects exerted by stigmasterol and sorafenib combination against MDA-MB-231 and MCF-7 breast cancer cells via assessing their impact on apoptosis, angiogenesis, and proliferation via elucidating NFκB-VEGF/BCL2 and ERK/caspase-3 pathways crosstalk.

According to the cell viability assay, sorafenib was a potent toxicant for both cell lines.

On the other hand, weak cytotoxicity was produced by stigmasterol. Similar cytotoxicity results were reported by previous breast cancer studies for either sorafenib [31] or stigmasterol [32].

Following exposure to the sorafenib and stigmasterol combination, proliferation was inhibited in both cell lines, as confirmed by low ki-67 levels. The suppression of cell proliferation is ascribed to the significant inhibition of NF-κB signaling, which controls cell cycle regulators [33].

The combination of stigmasterol and sorafenib also had a significant antiangiogenic effect, as evidenced by a decline in the expression of VEGF-A and its receptor VEGFR-2. This finding is explained by the inhibitory effect of sorafenib and stigmasterol combination on NF-kB signaling and hence down-regulation of VEGF expression [6, 7].

The stigmasterol and sorafenib combination group showed a significant reduction in the levels of anti-apoptotic protein Bcl-2 associated with a significant increase in caspase-3 activity. These outcomes were consistent with previous studies, which demonstrated that sorafenib had an effective apoptotic impact when combined with other agents on MDA-MB-231 cells [34] and MCF-7 [35]. Recent studies have also reported that stigmasterol can induce apoptosis in the cancer cell lines of ovarian [19], stomach [20, 21], liver [22], and gall bladder [23]. The enhancement of apoptosis is due to the inhibition of mitogenic NF-κB /VEGF signaling by stigmasterol and sorafenib combination [5, 7, 36]. This mitogenic pathway is responsible for the up-regulation of anti-apoptotic BCL-2 protein and apoptotic signals blocking [5, 7, 36].

Concerning ERK activity, a non-significant difference in the p-ERK activity was detected in the stigmasterol and sorafenib combination group compared to either sorafenib or stigmasterol alone. This contradicts a previous study which showed that stigmasterol results in a significant decrease in p-ERK levels [19]. This contradiction is partly attributed to the different cell lines with different genetic background in these studies, and may be due to the inhibition of the negative modulators of ERK, such as dual specific phosphatase (DUSP) due to stigmasterol and sorafenib treatment leading to activation of cytoplasmic ERK and hence induction of mitochondrial apoptosis [11]. It has been reported that p-ERK could promote cell death via the down-regulation of Bcl-2 and activation of caspase-3 [11].

5- Conclusion

From this study, we conclude that stigmasterol potentiates the chemotherapeutic efficacy of sorafenib through modulation of the crosstalk of the NF-κB/VEGF/BCL-2 pathway and ERK/Caspase-3 pathway.

In the future, it is necessary to conduct more research to analyze the influence of stigmasterol and sorafenib combination on other oncogenic signaling pathways.

6- Conflicts of Interest

The authors declare no conflict of interest.

7-Acknowledgements

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