The anti-tumor effect of stigmasterol on sorafenib treated human breast cancer cell lines

asmaa elshamy (asmaa.elshamy@pharm.dmu.edu.eg)
Damanhour University Faculty of Pharmacy

Gamal Omran
Damanhour University Faculty of Pharmacy

Mohammad Abd-Alhaseeb
Damanhour University Faculty of Pharmacy

Maha Houssen
Damanhour University Faculty of Pharmacy

Research Article

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Abstract

Background: Breast cancer is the most common invasive malignancy and the leading cause of tumor-related death among women globally. Excessive angiogenesis, sustained proliferation, and evasion of apoptosis are crucial for breast cancer progression. Sorafenib is a multi-kinase receptor inhibitor with anti-angiogenic activity. Stigmasterol is a phytosterol with anticancer activity. The aim of this study was to investigate molecular mechanisms of action of sorafenib and stigmasterol in two different human breast cancer cell lines and assess their combined impact on modulation of signaling pathways that control breast cancer pathogenesis.

Methods and results: MCF-7 and MDA-MB 231 cells were used as models of human breast cancer. MTT assay was used to assess cytotoxicity. Angiogenic VEGF/VEGFR-2/ERK/NF-kB signaling pathway was investigated using ELISA and RT-PCR techniques. Apoptotic markers (caspase-3, Bcl2) and a proliferation marker (Ki-67) were assessed using colorimetric and ELISA techniques. Sorafenib combined with stigmasterol increased caspase-3 activity and decreased Bcl2, VEGF-A, VEGFR-2, NF-kB and Ki-67 levels.

Conclusion: The combination of Sorafenib and stigmasterol may be a useful therapeutic regimen for breast cancer treatment. This combination may inhibit angiogenesis and promote apoptosis signaling.

Introduction

Breast cancer is the most commonly diagnosed cancer in women globally and is also an important cause of death [1]. Sustained proliferation and evasion of apoptosis are significant processes that contribute to breast cancer progression [2].

Angiogenesis is critical for progression and is associated with decreased survival [3]. Thus, angiogenic factors, such as vascular endothelial growth factor (VEGF) [3], are reasonable targets for therapy. This modulator induces angiogenesis through interaction with VEGF receptors (VEGFRs) and holds promise as a therapeutic approach for breast cancer treatment [3].

Sorafenib is a multiple tyrosine kinase inhibitor that inhibits a spectrum of receptor tyrosine kinases involved in angiogenesis, including VEGFR2, Flt-3, PDGFR-beta, and c-kit [4]. This agent also inhibits basal phosphorylation of downstream RAF/MEK/ERK signaling [4]. However, adverse effects, including drug resistance, are challenges of sorafenib treatment [4].

Many studies are ongoing to find alternative or adjuvant regimens to accompany sorafenib treatment. Several studies show that sorafenib in combination with adjuvant products is promising for treatment of multiple breast cancer in clinical trials [5, 6].

Many natural products have anti-tumor activity and are sufficiently potent for cancer therapy. These agents act via a variety of mechanisms. In addition, phytochemicals are helpful as adjuvant therapy to reduce the toxicity of chemotherapeutic drugs and to overcome multidrug resistance [7].
Stigmasterol is a phytosterol with a cholesterol-like chemical structure. It is commonly present in fruits and vegetables and is also consumed through different sources, such as plant oils and seeds, soybean, corn, peanuts, cereals, legumes and sunflower oils [8].

Stigmasterol displays a pro-apoptotic properties in treatment of ovarian [9], gastric [10, 11], liver [12] and gall bladder [13] cancers. It also inhibits ERK signaling in ovarian cancer [9] and VEGFR2 expression in cholangiocarcinoma [14].

The aim of the present study was therefore to investigate molecular mechanisms for the combination of sorafenib and stigmasterol on two different human breast cancer cell lines; and to assess modulation of signaling pathways that promote breast cancer pathogenesis.

**Materials And Methods**

**Chemicals**

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

**Drugs**

Sorafenib tosylate (≥98 % HPLC) and stigmasterol (~95%) were supplied by Sigma-Aldrich (St. Louis, USA). Drugs were dissolved in DMSO to prepare 10 mM sorafenib and 100 mM stigmasterol solutions. These stock solutions were stored at refrigerator temperature until use.

**Cell lines**

MCF7 and MDA-MB-231 are human breast cancer cell lines obtained from the Center of Excellence for Research in Regenerative Medicine and Applications (CERRMA, faculty of medicine, Alexandria University, Egypt).

**Cell cultures**

Cells were cultured as monolayers in T-75 tissue culture flasks containing DMEM with 10% FBS and 1% penicillin-streptomycin. Cells were incubated in a humidified incubator containing 5% CO2 at 37°C. Cells were passaged when they reached 80–90% confluence.

**Cell viability assessment (MTT assay)**

Cell viability and drug cytotoxicity were investigated using MTT assay [15]. Cells were seeded in 96-well plates containing 200 µL of complete medium/well then incubated overnight in 5% CO2 at 37°C. Medium was replaced with the same medium containing different concentrations of serially diluted drugs with not more than 0.1% DMSO. Concentrations of (3 µM, 6 µM, 12 µM, 24 µM, and 48 µM) were used for
sorafenib and (35 µM, 70 µM, 140 µM, 280 µM, and 560 µM) for stigmasterol. Plates were incubated for 48 hours. The medium was discarded and 50 µL of MTT solution (5mg/mL in phosphate buffered saline) was added and incubation continued for an additional 4 hours. MTT was removed and 50 µL DMSO was added to dissolve crystals. Absorbance was recorded with a microplate reader at a wavelength of 490 nm. Cell viability was expressed as percentages of untreated control wells.

**Experimental design**

Four groups of three replicate flasks of both MCF7 and MDA-MB-231 cells were cultured. Groups of flasks were assigned to different therapeutic regimens of sorafenib (18.6 µM for MCF7 and 21.5 µM for MDA-MB-231), stigmasterol (1000 µM for both cell lines) or a combination of both sorafenib and stigmasterol. A fourth group was kept untreated as a control. All cells were incubated for 48 hours before harvest.

**Preparation of cell lysate and protein quantification using BCA assay**

Cells were scraped from the surface of flasks using a cell scraper. Cell suspensions were then centrifuged at 600xg for 5 min and supernatants were discarded. Cells were re-suspended in phosphate buffered saline, centrifuged and supernatants were removed. RIPA Lysis Buffer (Catalog Number: AR0105, Boster Biological Technology, Pleasanton, USA) was added to cell pellets along with protease and phosphatase inhibitors, then incubated on ice for 30 minutes. Cell lysates were centrifuged at 14000xg for 10 minutes and supernatants were transferred to new tubes for further analysis [16].

Total protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Catalog number: 23225, Thermo fisher scientific, USA) following the manufacturer's instructions.

**Biochemical analyses**

**Proliferation markers**

Ki-67 was analyzed using Human Ki67 Simple Step ELISA® Kit (ab253221, Abcam, UK) following the manufacturer’s instructions.

**Angiogenic signaling markers**

VEGF, VEGFR-2, NF-kB and p-ERK levels were analyzed using Human VEGF Quantikine ELISA Kit (Catalog Number: DVE00, Bio-Techne, USA), Human VEGF Receptor 2 Simple Step ELISA® Kit (ab213476, Abcam, UK), Human Nuclear Factor Kappa B (NF-kB) ELISA Kit (Catalog No: MBS450580, MyBioSource, USA) and Human pERK1/2 (Phospho Extracellular Signal Regulated Kinase 1/2) ELISA Kit (Catalog No: MBS2511875, MyBioSource, USA) respectively following the manufacturers’ instructions.

**VEGFR-2 mRNA gene expression**

Gene expression of VEGFR2 was assessed using one step real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using a miRNeasy Mini Kit (Cat. No: 217004, Qiagen,
USA) following the manufacturer’s instructions. PCR reactions were conducted using a Rotor-Gene SYBR Green RT-PCR Kit (Cat. No: 204174, Qiagen, USA) following the manufacturer’s instructions. Reverse transcription was performed at 55°C for 10 min followed by PCR initial activation step at 95°C for 5 min, 35 PCR cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 10 s [17].

(β Actin) was used as a housekeeping gene with forward primer sequence: 5’ACCATGGATGATGATATCGC3’ and reverse primer sequence 5’CATAGGAATCCTTCTGACCCCA3’. VEGFR2 primer sequences were F: 5’CCTGGGAAATCAGACGACAA3’ and R: 5’CCGGTTCCCCATCCTTCAATA3’. Each preparation was analyzed in triplicate and relative quantification was used for comparisons among results.

**Apoptosis markers**

Caspase-3 activity was determined using a Caspase-3 Colorimetric Assay Kit, (Sigma Aldrich, USA) (Product Code CASP-3-C) and Bcl-2 levels were assessed using Bcl-2 Human ELISA Kit ((ab119506), Abcam, UK). Analyses were performed following the manufacturers’ instructions.

**Statistical analysis**

Results are expressed as the mean ± standard deviation of the mean (SD) of three samples, each performed in triplicate. One-way analysis of variance followed by Tukey post hoc tests were used for multiple comparisons through Graph Pad Prism Software (version 8.0.2) .Statistical significance of differences between treatment results was fixed at $p \leq 0.05$.

**Results**

**Cell viability assessment (MTT) assay**

Sorafenib inhibited the growth of both MCF7 and MDA-MB-231 cells, with an IC$_{50}$ of 18.6 µM for MCF7 and 21.5 µM for MDA-MB-231 (Fig. 1). Conversely, stigmasterol inhibition of cell growth was weak and no IC$_{50}$ could be estimated because 70% cell viability was observed at the highest concentration. One thousand µM was adopted as a therapeutic concentration for subsequent work.

**Biochemical analyses**

**Effect of sorafenib and stigmasterol on cell proliferation marker ki67**

Sorafenib exposure of MCF7 cells was the only single treatment that significantly reduced Ki 67 levels. However, Ki67 levels in MCF7 and MDA-MB-231 cells were significantly reduced by exposure to sorafenib and stigmasterol in combination compared to either agent alone ($p \leq 0.05$) (Fig. 2).

**Effect of sorafenib and stigmasterol on angiogenesis**
Both MCF-7 and MDA-MB 231; showed a significant reduction in p-ERK, NF-kB, VEGF, and VEGFR-2 levels in cells exposed to sorafenib, stigmasterol, or their combination. Combined treatment showed significantly lower levels of NF-kB, VEGF and VEGFR-2 compared to either sorafenib or stigmasterol alone \((p \leq 0.05)\) (Fig. 3).

**Effect of sorafenib and stigmasterol on VEGFR-2 gene expression**

VEGFR-2 mRNA gene expression was significantly reduced by sorafenib, stigmasterol, or their combination in both MCF-7 and MDA-MB 231. A significantly greater response in expression was observed for combined treatment than for either sorafenib or stigmasterol alone \((p \leq 0.05)\) (Fig. 4).

**Effect of sorafenib and stigmasterol on caspase-3 activity and Bcl-2 level as apoptotic markers**

Caspase-3 activity and Bcl-2 levels showed a significant increase in caspase-3 activity and a significant decrease in Bcl2 levels in both cell types exposed to sorafenib, stigmasterol, or their combination compared to control. Again, the response to combined treatment was significantly greater than responses to sorafenib or stigmasterol alone \((p \leq 0.05)\) (Fig. 5).

**Discussion**

Breast cancer is a substantial global health issue and is associated with the highest number of cancer related deaths in women [1].

A continuous need for new therapeutic approaches to breast cancer reflects its highly heterogeneous nature with many subtypes that can develop resistance to traditional treatment [18].

The aim of the present study was twofold. The first was to investigate the molecular chemotherapeutic effect of sorafenib as a multi-tyrosine kinase inhibitor in comparison to stigmasterol, a phytosterol with anticancer activity, on different signaling pathways in MCF-7 and MDA-MB-231 human breast cancer cells. The second was to assess the impact of a combination of sorafenib and stigmasterol on proliferation, angiogenesis, and apoptosis signaling involved in promoting breast cancer progression.

Cytotoxicity assays showed that sorafenib is a potent toxicant to cells in both lines, but stigmasterol cytotoxicity was weak; an \(IC_{50}\) was not estimated since even high concentrations did not cause 50% lethality. Previous studies reported similar results for sorafenib [19] and stigmasterol [20] cytotoxicity in breast cancer.

A combination of sorafenib and stigmasterol caused a decrease in the proliferation marker, Ki-67. This effect is ascribed to the inhibition of ERK signaling [9, 19, 21]. Inhibition of ERK prevents activation of downstream transcription factors and cell cycle regulators leading to diminishing cell proliferation [21].
A significant antiangiogenic effect was mediated by both compounds individually. Levels of p-ERK and its downstream transcription factor, nuclear factor kappa B (NF-kB) were reduced. Both agents also induced decreased expression of angiogenic mediator, VEGF-A, and its receptor, VEGFR-2.

A further decrease in NF-kB, VEGF-A and VEGFR-2 levels was observed in cells treated with a combination of agents. The effect of sorafenib on p-ERK is attributed to its inhibitory effect on RAF [4] and this effect was observed in a recent study using MCF-7 and MDA-MB 231 cells [19]. Stigmasterol inhibited the phosphorylation of ERK1/2 in a recent ovarian cancer study [9]. NF-kB [22, 23] and other transcription factors, such as FoxM1 [21], were suppressed; as a result of ERK inhibition. Downregulation of NF-kB in this study is consistent with previous studies on sorafenib [24] and stigmasterol [25]. Inhibition of transcription factor, FoxM1, leads to downregulation of VEGF. This factor binds directly to Forkhead binding elements (FHRE) of the VEGF promoter that activates VEGF expression [21]. VEGF was also downregulated following the inhibition of NF-kB in MCF-7 and MDA-MB-231 cells [26, 27].

Both sorafenib and stigmasterol induced caspase-3 activity and decreased levels of the anti-apoptotic protein, Bcl-2. These responses were enhanced by combined treatment. Sorafenib exerted an efficient apoptotic effect in combination with other agents in a previous study carried on MCF-7 [28] and MDA-MB-231 cells [29]. Induction of apoptosis by stigmasterol was also reported in recent studies in ovarian [9], gastric [10, 11], hepatic [12] and gall bladder [13] cell lines. Apoptotic effects are ascribed to inhibition of both RAF-MEK-ERK and PI3K-AKT signaling by both agents [9, 10, 30]. These pathways are responsible for phosphorylation and inactivation of several factors, including pro-apoptotic Bad protein and caspase-9 [31, 32], leading to caspase-3 inhibition, and blocking of apoptotic signals. Apoptosis is also attributed to downregulation of NF-kB. This factor upregulates anti-apoptotic proteins, including Bcl-2, and transactivates inhibitors of apoptosis proteins (IAPs) [31]. In addition, reduction of anti-apoptotic Bcl-2 protein by the combination of sorafenib and stigmasterol promotes the release of cytochrome c and initiation of the intrinsic apoptotic cascade [31].

Thus, the combination of sorafenib and stigmasterol may prove useful for breast cancer treatment. Future studies are warranted to investigate the impact of this combination on several signaling pathways that mediate breast cancer pathogenesis.

**Declarations**

**Acknowledgement**

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**Authors' contributions**

Asmaa Elshamy: conducting all the practical experiments, writing the first paper drafts, collecting review of the literature, corresponding author. Gamal Omran: revising the final version of the paper. Mohammad
Abd-Alhaseeb: supervise the practical work, revising all paper drafts. Maha Houssen: Design research idea and construct the research plan, revising the research results, sharing in authoring all paper drafts, revising all paper drafts, collecting review of literature, supervise all work.

Statements and Declarations

Funding

Compliance with ethical standards

The study was approved by the Ethical Committee of the Faculty of Pharmacy, Damanhour University (Ref.no 1219PB14)

Conflict of interest

The authors declare that they have no conflict of interest.

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**Figures**
Figure 1

![Graph showing MTT results for MCF7 and MDA cells with sorafenib concentration on the x-axis and % cell viability on the y-axis.]

Figure 1
Figure 2
Figure 3

(a) VEGF levels in MCF-7 and MDA-MB-231 cells treated with control, Sorafenib, Stigmasterol, or Combination.
(b) VEGFR-2 levels in MCF-7 and MDA-MB-231 cells treated with control, Sorafenib, Stigmasterol, or Combination.
(c) P-ERK levels in MCF-7 and MDA-MB-231 cells treated with control, Sorafenib, Stigmasterol, or Combination.
(d) NF-kB levels in MCF-7 and MDA-MB-231 cells treated with control, Sorafenib, Stigmasterol, or Combination.
Figure 4

Figure 5

Figure 5