

RESEARCH ARTICLE

Stem Cell Factor Antibody: Effective Manipulation of Antihormonal Therapy in Resistant Human Breast Cancer *In Vitro*

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ABSTRACT

Tamoxifen is the most commonly used drug in the treatment of breast cancer via blocking the estrogen receptor pathway. However, the use of Tamoxifen is limited by intrinsic and acquired resistance, which may be associated with the de-regulation of the kinase protein expression or an increase in multiple drug resistance (MDR) expression. Two breast cancer cell lines, wild type MCF7 WT (sensitive, i.e., ER⁺) and MDA-MB-231 (resistant, i.e., ER⁻), were used. Expression of P-glycoprotein (Pgp) was measured, the cells were treated with 4-hydroxy tamoxifen in the presence or absence of anti-stem cell factor, apoptosis protein (Annexin V) was measured and Influx/efflux rates were monitored by using Technetium^{99m} methoxyisobutylisonitrile (^{99m}Tc-Sestamibi-MIBI) at different time intervals. Results showed positive expression of Annexin V in MDA-MB-231 and MCF7/WT, and the effect of blocking of the stem cell factor showed an increase in the drug accumulation within the MDA-MB-231 cell line. In conclusion, this study showed that the anti-stem cell factor enhances the effectiveness of antihormonal therapies determined by ^{99m}Tc-MIBI. These findings may have implications for the use of anti-stem cell factor with antihormonal therapy in ER-negative breast cancer in order to overcome drug resistance and improve the outcome.

Keywords: Antibody, Antihormonal therapy, Human breast cancer.

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INTRODUCTION

Breast cancer is the most commonly diagnosed disease and the leading cause of cancer death in women worldwide.¹ Tamoxifen is still the therapy of choice for ER⁺ breast cancer. However, over 30% of breast cancer patients with ER⁺ fail to respond to tamoxifen, whereas the responding patients may finally progress to a resistant phenotype.^{2,3}

Despite the significant improvements in cancer treatment, resistance to therapeutic agents still represents a significant issue for optimal clinical management, partially due to the decrease in intracellular drug accumulation, i.e., increase the efflux rate.^{4,5} In breast cancer, a significant correlation was indicated between intercellular drug accumulation and acquired resistance to tamoxifen. Studies suggested an association between the presence of P-glycoprotein (Pgp) and ineffective therapeutic medication resulting in poor prognosis. Therefore, Pgp expression might play a role in tamoxifen resistance.⁶⁻⁸

On the other hand, Previous studies showed evidences of the role of the signaling transduction pathways such as

mitogen-activated protein kinase (MAPK), c-Jun NH₂-terminal kinase (JNK), p38, cyclic adenosine monophosphate-dependent protein kinase, phosphatidylinositol 3-kinase and protein kinase C signaling pathways in the regulation of the ATP-binding cassette (ABC) transporters.⁹ The ABC proteins are part of the largest family of transmembrane proteins. These proteins function as energy-dependent and transport a variety of molecules/substrates across cellular membranes.¹⁰ The expression of these transport proteins is implicated in pumping the drugs out of the cells, which prevents intracellular anticancer drug accumulation. This drug accumulation causes the development of multiple drug resistance (MDR), which is considered as one of the cellular pathways involved in resistance to anticancer drugs. These protein transporters include ABCB1 (P-glycoprotein), ABCC1 (MRP1), ABCC2 (MRP2), ABCC4 (MRP4), ABCG2 (BCRP) and the lung resistance protein (LRP).^{11,12}

To provide the basis for more effective combined molecular/endocrine therapy regimen in breast cancer patients, many efforts have been made for better understanding of tamoxifen

resistance mechanism in relation to ER expression, signal transduction pathway and co-regulatory proteins.¹³ Therefore, it is become necessary to switch to new treatment regimens to overcome MDR phenotype and increase drug accumulation i.e., inhibit drug efflux rate to sensitize cancer cells to therapeutic agents.¹⁴

C-kit (CD-117) is a proto-oncogene trans-membrane tyrosine kinase receptor (KIT), mainly found express in primitive haematopoietic cells, mast cells, melanocytes, and breast glandular epithelial cells, and is detected in the testicular and ovarian interstitial cells.¹⁵⁻¹⁷

Stem cell factor (SCF), which also known as (Kit Ligand-KL, mast cell growth factor-MCGF and steel factor -SLF), is a haematopoietic cytokine present in both soluble and trans-membrane forms. SCF has a wide range of biological activities in both normal and malignant tissues; it acts as a growth factor and regulates proliferation and differentiation.^{18,19}

The binding of the SCF and Kit induces receptor dimerisation, resulting in enhancement of the tyrosine-kinase activity and inducing signal transduction, which leads to biological activities such as cell apoptosis, proliferation, differentiation, adhesion, and motility.^{20,21} Ulivi and colleagues indicated a high expression of C-Kit/CSF in invasive breast cancer compared with the expression in normal breast cancer.²² Blocking of SCF using anti stem cell factor (ASCF) enhanced the response to Adriamycin drug resulting in apoptosis of the leukaemia cell line.²³

From therapeutic perspective, the study of the c-Kit/CSF signaling pathway in relation to Pgp function becomes necessary for the development of targeted therapies to reduce drug efflux rate and to overcome resistance phenomena in diseases such as breast cancer

Therefore, the goal of the present study was to enhance tamoxifen efficacy in ER-negative breast tumors using stem cell factor-antibody (anti-SCF).

MATERIALS AND METHODS:

All the used reagents in this study were obtained from Sigma Aldrich, UK unless stated. All used cells were undergone between 15–20 passages. Cells were incubated whenever mention at 37°C in air/5% CO₂ incubator, unless stated.

Cell Culture and Growth

Tamoxifen sensitive cell line MCF7/WT and ER-negative MDA-MB-231 cell line obtained from the European type culture collection (ECACC). Cells were grown in complete tissue culture medium consisting of Iscove's Modified Dulbecco's Medium, IMDM supplemented with an antibiotic solution (100 units/ml of penicillin, and 0.1 mg/ml of streptomycin) and 10% fetal calf serum (FCS), and placed in the incubator.

Flow Cytometric Analyses for ATP-binding cassette Transporter

From each cell line (1×10⁶ cells/mL), cells were fixed in 1 ml of ice-cold methanol, permeabilized using 100 μL of 0.1% triton -X100 in PBS, solution. The cells were labeled with

P--glycoprotein (Pgp) monoclonal antibody (Santa Cruz Biotechnology, Inc., USA) and diluted in permeabilized solution (at a final concentration according to the manufacture's recommendation) Finally the cells were incubated with fluorochrome-labeled secondary antibody, which had been prepared in 3% BSA/PBS according to the manufacture's recommendation and cells were incubated at 4°C in the dark for 30 minutes, then cell were washed three times according to the washing procedure and resuspended in ice-cold PBS, 3% BSA and immediately analyzed using flow cytometry. (Beckman Coulter Diagnostics, UK).

Apoptosis Assessment

Cell apoptosis was detected by staining with FITC-Annexin V. Cells lines were seeded in 25 cm² tissue culture flasks in the incubator. After 24 hours, the cells were treated with anti stem cell factor (ASCF) and/or stem cell factor (SCF) in combination with 4-OH at 0.4 ug/mL, 100 units/ml and 17.5 uM respectively, and were incubated again for 48 hours. Quantitative expression of Annexin V was measured using Annexin V Elisa kit (Abnova-Taiwan), according to the manufacturer's instructions.

^{99m}Tc-Sestamibi (MIBI) accumulation

The cultured cells were harvested and re-suspended at a concentration of 1×10⁶ cells/ml in fresh medium and were incubated for 2 hours with ASCF and/or 4-OHT. The ^{99m}Tc-Sestamibi (MIBI) experimental protocol was previously reported, briefly; single-cell suspensions at 10⁶ cells per ml incubated with stirring in a 37°C water bath for 5 min to allow equilibration²⁴ (Muzzammil *et al.*, 2001). ^{99m}Tc-sestamibi cardio lite kit (IBA molecular, UK was added at a final concentration of 0.5 MBq/mL, samples were taken after at 0, 30, 60, and 90 min. a. duplicate aliquots of 200 were removed from each tube and transferred to 5-ml microcentrifuge tubes containing 800 ice-cold PBS and centrifuged at 14000 g for 2 min. The supernatant was aspirated, and the cell pellet was washed with 1 mL ice-cold PBS, the pellet was assayed in duplicate for radioactivity using a gamma counter (Wizard2-PerkinElmer,UK). The accumulation ratio was calculated, the ratio of radioactivity inside the cell to that outside (in/out).

Statistical analysis

Analyses were performed using the statistical analysis system IBM SPSS. P-values were determined using one and two way ANOVA. The data were presented as mean ± standard error (SE) with a value of p < 0.05 were considered to be statistically significant and p < 0.01 as a highly significant.

RESULTS

P-glycoprotein (Pgp) expression

Cell surface P-glycoprotein expression was detected by flow cytometric analysis. Cell surface P-glycoprotein staining was positive in the MDA-MB-231 cell line. However, this expression was minimal or absent in MCF-7/WT. These results indicated that the drug expulsion mechanism is relatively active in the ER-negative breast cancer cells and not in the ER-positive breast cancer cell line (control).

Annexin V and SCF neutralization

The effects of ASCF with or without 4-OHT on inducing apoptosis were determined via quantitative assessing of annexin V expression using ELISA. A significant expression of annexin V was indicated in MDA-MB-231 and ER-positive MCF7/WT cell lines after treating the cells with ASCF and 4-OHT (Figure 2).

Technetium (99mTc) sestamibi (MIBI)

The specificity of Tc99m for ABC transport assays was performed in the two breast cell lines MCF7/WT, MDA-MB-231, cells incubated with ASCF alone and with 4-OHT. The effect of the ASCF on MDA-MB-231 increasing the accumulation was significant; when the cells incubated with both ASCF and tamoxifen, the accumulation increased with time ($p < 0.05$), Figure (4). On the other hand, the high rate of the drug accumulation hasn't affected by ASCF in the ER-positive MCF7/WT, shown in Figure 3.

DISCUSSION

Most of the studies suggest that the mode of action of tamoxifen is mediated by competitive inhibition of estrogen binding to ER.²⁵ However, some studies showed that tamoxifen could

mediate its effect via ER independent mechanism, involving molecular, such as protein kinase c, TGFβ, and MAPKs.²⁶

Therefore, testing the new and more specific molecular-targeted therapeutic approaches in ER⁺ breast cancer cells can offer a new concept for a more efficient combined molecular/endocrine therapy regimen and may improve the treatment outcome.

It is well-documented fact that the presence of multidrug resistance protein (Pgp) the present study and despite differences in the presence and absence of ER expression, in MCF7/WT and MDA-MB-231 cells, which is inversely correlated with the presence of multidrug resistance protein (Pgp) as shown in Figure 1, results shows the ability of the ASCF to sensitize MDA-MB-231 cells to the anti-proliferative effects of tamoxifen and an increase in the drug accumulation in response to tamoxifen when were treated with a combination of ASCF and 4-OHT, Figures 2, 3 and 4.²⁷

This observation may be explained by the role of c-Kit in the downstream signaling molecules through an interaction with stem cell factor (SCF), this interaction could play an

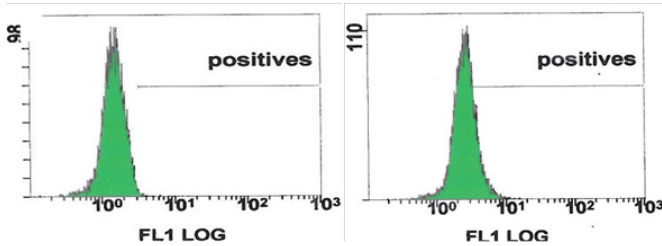


Figure 1: The breast cancer cell lines used for indirect immunophenotyping of Pgp using flow cytometry. (a) Immunophenotyping of Pgp showing (<1%) in MCF7/WT. (b) Immunophenotyping of Pgp showing (24.9%) in MDA-MB-231.

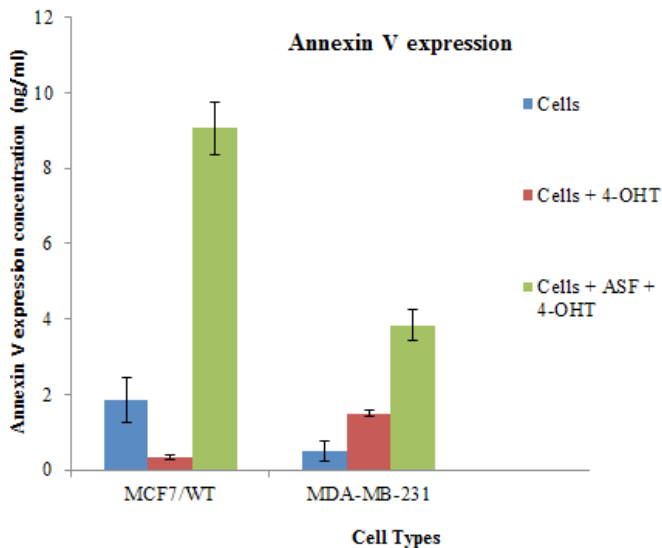


Figure 2: Effect of ASCF and SCF on Annexin V expression in the breast cancer cell lines (MCF/WT, and MDA-MB-231). All the cells were treated with 4-OHT alone and with ASCF. Results (n=3) are expressed as the mean value, ±SE. * $p < 0.05$.

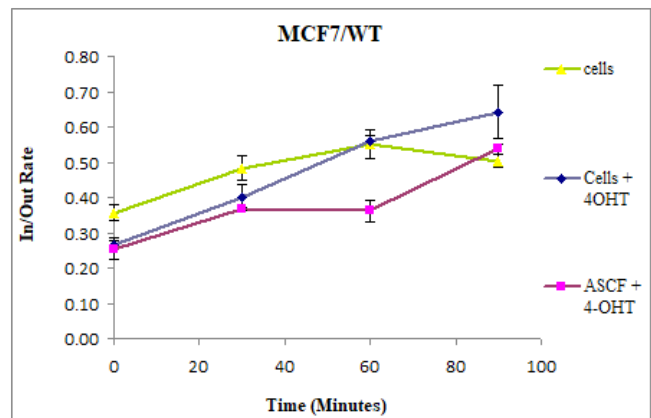


Figure 3: Drug accumulation rate (In/out rate) for MCF7/WT cells treated with anti stem cell factors and incubated with 4-OHT. Data represent the mean value from three independent experiments (n=3) ±SE and incubated with 4-OHT. Data represent the mean value from three independent experiments (n=3) ±SE.

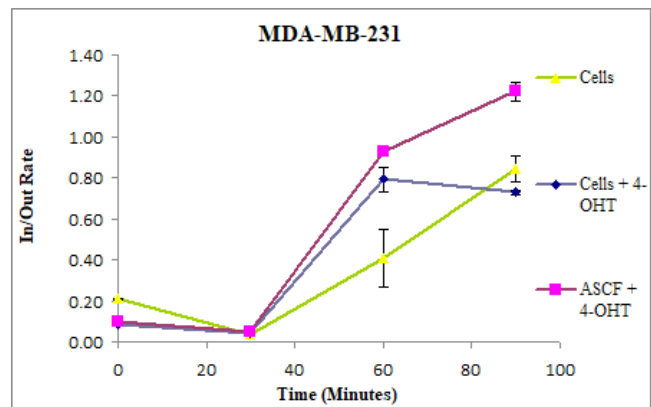


Figure 4: Drug accumulation rate (influx/efflux rate) for MDA-MB-231 cells treated with anti stem cell factors and incubated with 4-OHT. Data represent the mean value from three independent experiments (n=3) ±SE.

oncogenic role via regulating cell proliferation, anti-apoptosis, tumor reoccurrence and metastasis, which subsequently lead to an independent stimulation of the cellular functions.^{28,29}

The importance of c-kit and its ligand, stem cell factor in the enhancement of proliferation and invasion via the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, has been demonstrated in c-Kit positive colorectal cancer.³⁰ Also, the stem cell factor is known as a tumor-derived factor, and previous studies have observed the expression of SCF and its receptor c-Kit in breast cancer.²²

Nevertheless, ASCF elevation of apoptosis in MDA-MB-231 is in agreement with the role of c-Kit and SCF in activating the transduction signaling cascade and the effects of these signaling cascades on ER phosphorylation as an independent mechanism of action in tamoxifen-resistant breast cancer.³¹⁻³⁴ Furthermore, it is well documented that SCF activates Erk1/2 and p38 mitogen-activated protein kinase (MAPK) and that the blocking of SCF might lead to a reduction MAPK activity, and that might explain the growth inhibition.³⁵

In the present study, the effect of ASCF in enhancing the response of tamoxifen was carried out via determining the expression of annexin V using ELISA. A significant result ($p < 0.005$) was indicated when MDA-MB-231 and MCF/WT cells were treated with a combination of ASCF and 4-OHT. Cumulatively, these data suggest that ASCF might enhance the response to tamoxifen in ER-negative tamoxifen-resistant breast cancer (ER⁻) and via involvement in the signaling cascade., which is in agreement with the previously published study of overcoming resistance to endocrine therapy and enhancing apoptosis by blocking of the c-kit and inhibition of signal transduction.^{36,37}

The results from this study indicated that SCF neutralization could play a significant role in reducing the growth rate in MDA-MB-231 compared with MCF7/WT. Results from the present study are in agreement with previous studies on different tumor types such as pancreatic cancers and melanoma.^{38,30}

For any cancer therapy, treatment efficiency determined by the delivery of high doses of the drug to tumor sites at the present study, the effects of the ASCF on the drug accumulation were evaluated using technetium ^{99m}Tc sestamibi uptake rate. Results showed a significant increases in influx/efflux rate in the MDA-MB-23 cell line, as shown in Figure 4 when treated with ASCF, also an increase of the uptake was noticed in the control cell lines, but to a lesser (non-significant) degree., this is indicating a potential role of the ATP-binding cassette (ABC) protein in drug resistance.^{39,40}

Interestingly, our study demonstrated that the inhibition of the stem cell factor associated with an improved tumor drug uptake and a concomitantly enhanced therapeutic effect of the tamoxifen, this has been noticed in the increasing of ^{99m}Tc-MIBI uptake, which is predicting the tumor response to the drug.⁴¹

These data suggest that the interference with SCF/c-Kit loop could be novel approaches to address tamoxifen resistance phenomena in the clinical management of breast cancer and to

consider the pharmacologic utilization of both ER-dependent and ER-independent antitumor activities of tamoxifen and to avoid the multi drugs resistance development. Also, in this study, for the first time ^{99m}Tc-MIBI technique has been used to predict the drug uptake before and after the blocking of SCF in the breast cancer cell line.^{42,43}

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