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The effects of low-dose sorafenib on epithelial-mesenchymal transition and multidrug resistance markers in HepG2 cell line

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ABSTRACT

Objectives: Sorafenib is an orally administered tyrosine kinase inhibitor in hepatocellular cancer. Low sorafenib concentrations are attained during pharmacotherapy due to pharmacokinetic profile and patient inadherence. Resistance to treatment is a limitation to improving survival. Underlying mechanisms include epithelial-mesenchymal transition. The aim of the study was to evaluate epithelial-mesenchymal transition and multidrug resistance-related parameters in HepG2 cells following low-dose and short-term sorafenib treatment.

Methods: Epithelial-mesenchymal transition and multidrug resistance-related markers were examined by quantitative PCR, flow cytometry, and confocal laser scanning microscopy.

Results: An increase in epithelial marker E-cadherin and downregulation of mesenchymal markers Vimentin and Snail1 were detected by gene expression analysis. While P-glycoprotein expression increased, multidrug resistance protein 1, and breast cancer resistance protein mRNA levels did not alter after sorafenib treatment. The accumulation of the ABC transporter substrate rhodamine 123 in the cells increased following the treatment, corresponding to a less efficient efflux of rhodamine 123 and a possible effect on other transporters and mechanisms.

Conclusions: The results indicate a protective effect of sorafenib against epithelial-mesenchymal transition and upregulation in P-glycoprotein expression, which is, however, not sufficient to cause less intracellular rhodamine 123 accumulation. The effects of low-dose and short-term sorafenib on epithelial-mesenchymal transition and multidrug resistance-related markers might contribute to enlightening new treatment strategies in hepatocellular cancer

Keywords: Sorafenib, hepatocellular cancer, HepG2, multidrug resistance, epithelial-mesenchymal transition

Sorafenib is an orally administered tyrosine kinase inhibitor that increased overall survival in hepatocellular cancer (HCC). After oral intake, peak plasma concentrations are achieved within 3 hours, half-life varies between 25-48 hours [1]. Steady-state plasma concentrations are achieved in seven days. The drug

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concentration is reduced by 29% when it is taken with high-fat meals. Sorafenib is highly protein-bound (99.5%) [2], which leads to drug-drug interaction potential with lower sorafenib concentrations.

Sorafenib therapy might exert adverse effects. Gastrointestinal, dermatological, and cardiovascular adverse effects are frequently reported [3]. Therefore, dose reduction is considered an applicable strategy in clinical practice [4]. As therapeutic drug monitoring is not established for sorafenib, dose adjustment due to toxicity is the standard approach [5]. Moreover, though it is the best pharmacological approach, most HCC cases develop resistance to sorafenib therapy. Possible underlying mechanisms include epithelial-mesenchymal transition (EMT) [1].

EMT is defined as the transformation of the epithelial cells in cells with a mesenchymal phenotype. E-cadherin, Vimentin, N-cadherin, Twist, and Snail1 are prominent markers [6]. The loss of E-cadherin, acquisition of Vimentin, and upregulation of Snail-1 are reported in the progression of various cancers [7]. A worse prognosis is correlated with Twist and Snail expression in about 40%-70% of HCC cases with adherens junction disruption [8].

Multidrug resistance (MDR) is a multifactorial process, where cancer cells are unresponsive to the treatment. Increased drug efflux, decreased drug uptake, sequestration of drugs, changes in drugs metabolism, altered expression of non-coding RNAs, inhibition of apoptosis, alterations in the tumor environment, and expression of cancer stem cell (CSC) markers are considered among the mechanisms of MDR [9]. The ATP-binding cassette (ABC) transporters are membrane transport proteins, responsible to remove the anti-neoplastic drugs from the cells to achieve insufficient intracellular concentration and bioavailability. Twelve members of this large family are highly expressed in normal and neoplastic hepatocytes [10]. P-glycoprotein (ABCB1, MDR1, P-gp), multidrug resistance protein 1 (ABCC1, MRP1), and breast cancer resistance protein (BCRP) are the prominent efflux transporters associated with chemotherapy resistance and have been reported to impact the clinical outcomes in HCC treatment with sorafenib [11]. Inhibiting efflux transporters is a major strategy to resensitize the HCC cells and increase the response to sorafenib [12].

The presence of EMT within tumors is linked to CSCs that are also implicated in drug resistance [13]. Previously, our group observed that low-dose sorafenib (4 µM) treatment results in low cytotoxicity in HepG2 cells, yet also induces CSC-related changes [14]. This study is based on the known link between the CSCs and the occurrence of EMT and MDR within tumors [13, 15]. The purpose of the study is to investigate the effect of low-dose and short-term sorafenib (4 µM) treatment on EMT and/or MDR-related markers by gene expression analysis and functional tests in HepG2 cells.

METHODS

Cell Culture and Sorafenib Treatment

HepG2 cells (passage #15; American Type Culture Collection, USA) were maintained in Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, and % 1 (v/v) penicillin (10000 U/mL) and streptomycin (10 mg/ml) at 37 0C in a humidified incubator with 5% CO_2.
Sorafenib (LC Laboratories, USA) was dissolved in dimethyl sulfoxide to prepare a 10 mM stock. The cells were treated with 4 µM sorafenib for 72 hours, which was shown to be associated with approximately 75% cell proliferation with the cell counting kit-8 (CCK-8) assay previously by our group [14].

Total RNA Isolation, Reverse Transcription and Quantitative PCR (RT- qPCR)

Total RNA was isolated following sorafenib treatment for 72 hours by using GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. After confirming the RNA integrity, cDNA was synthesized from 1 µg total RNA with oligo dT primers and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). QPCR was carried out with RealQ Plus Master Mix Green Without ROX (Ampliqon, Denmark) and the gene-specific primers for detection of E-cadherin, Vimentin, Snail1, P-glycoprotein (ABCB1, MDR1, P-gp), multidrug resistance protein 1 (ABCC1, MRP1), breast cancer resistance protein (ABCG2, BCRP; aforementioned primers from BM Lab, Turkey) mRNA levels and GAPDH as an internal control (Thermo Scientific) in a LightCycler® 96 instrument (Roche Diagnostics International, Switzerland). Delta

delta Ct ($2^{-\Delta\Delta Ct}$) relative quantitation method was employed for quantification of gene expression [16].

Gene-specific primers are as following: 5'-CACTATGCCGCGCTCTTTC-3' and 5'-GGTCGTAGGGCTGCTGGAA-3' for Snail [17]; 5'-AGGCAAAGCAGGAGTCCACTGA-3' and 5'-ATCTGGCGTTCCAGGGACTCAT-3' for Vimentin (#HP206907, OriGene Technologies, USA); 5'-GCCTCCTGAAAAGAGAGTGGAAG-3' and 5'-TGGCAGTGTCTCTCCAAATCCG-3' for E-Cadherin (#HP207683, OriGene Technologies, USA); 5'-GGGAGCTTAACACCCGACTTA-3' and 5'-GCCAAAATCACAAGGGTTAGCTT3' for P-gp; 5'-TGTGGGAAAACACATCTTTGA-3' and 5'-CTGTGCGTGACCAAGATCC3' for MRP1; 5'-AGATGGGTTTCCAAGCGTTCAT-3' and 5'-CCAGTCCCAGTACGACTGTGACA-3' for BCRP. GAPDH primers were included in the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

Determination of Rhodamine 123 (Rh123) Accumulation by Flow Cytometry

The cells were treated with sorafenib for 72 hours. Next, they were trypsinized and centrifuged at 500 g for 5 minutes. After washing with phosphate-buffered saline (PBS), 10^6 cells per data point were resuspended in DMEM at pH 7.8 and 37 °C, containing Rh123 (Sigma-Aldrich). The cells were loaded with Rh123 at a final concentration of 0.2 µg/mL (0.53 µM) at 37 °C for 30 minutes in a water bath and taken into ice at the end of the incubation to terminate the loading. After chilling in ice, the cells were washed twice with ice-cold DMEM, pH 7.4. Finally, they were resuspended in ice-cold DMEM, pH 7.4 for measuring the Rh123 fluorescence in FL-1 channel in an Accuri C6+ flow cytometry (BD Biosciences, USA). The tubes were kept in ice during the measurements. A minimum of 20,000 events were recorded and Rh123 median fluorescence values were used for generation of the bar graphs.

Determination of Rhodamine 123 (Rh123) Accumulation by Confocal Laser Scanning Microscopy

The cells were harvested with trypsinization, counted and seeded at a concentration of 1×105 cells to 24-well plates containing autoclaved coverslips. After an overnight incubation, sorafenib was added to the cells and the cells were further incubated for 72 hours. Next, they were washed with PBS and incubated with Rh123 (final concentration of 1 µM). After washing with PBS, they were counterstained with 1 mg/mL of Hoechst 33258 (Thermo Scientific) and the images were obtained with a Zeiss LSM 700 confocal scanning microscope (Germany).

Statistical Analysis

All data are expressed as the mean ± standard deviation. Student's t-test was used the compare the groups. GraphPad Prism V.8.01 (GraphPad Software, USA) was employed for generating the bar graphs and performing the statistical analyses. An α of 0.05 was used as the cut off for significance.

RESULTS

Effects of Low-Dose Sorafenib Treatment on EMT-Related Gene Expression

To investigate whether low-dose sorafenib treatment alters the expression EMT-related genes and the cell migration, we analysed the mRNA levels of Vimentin, Snail1 and E-cadherin. In response to 4 µM sorafenib

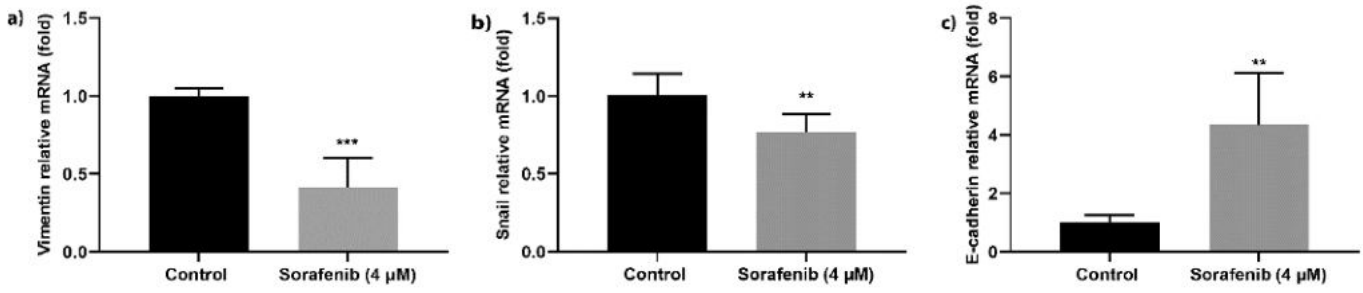

Fig. 1. Gene expression levels of (a) Vimentin, (b) Snail1 and (c) E-cadherin in response to 4 µM sorafenib treatment for 72 hours. Student's t-test was used to find significance. **$p < 0.01$, ***$p < 0.001$ vs control group.

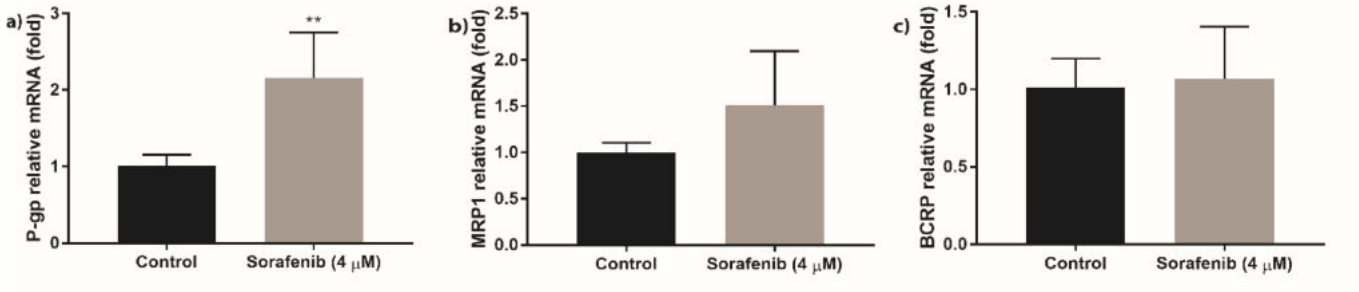

Fig. 2. Gene expression levels of (a) P-gp, (b) MRP1 and (c) BCRP in response to 4 μM sorafenib treatment for 72 hours. Student's t-test was used to find significance. **$p < 0.01$ vs control group.

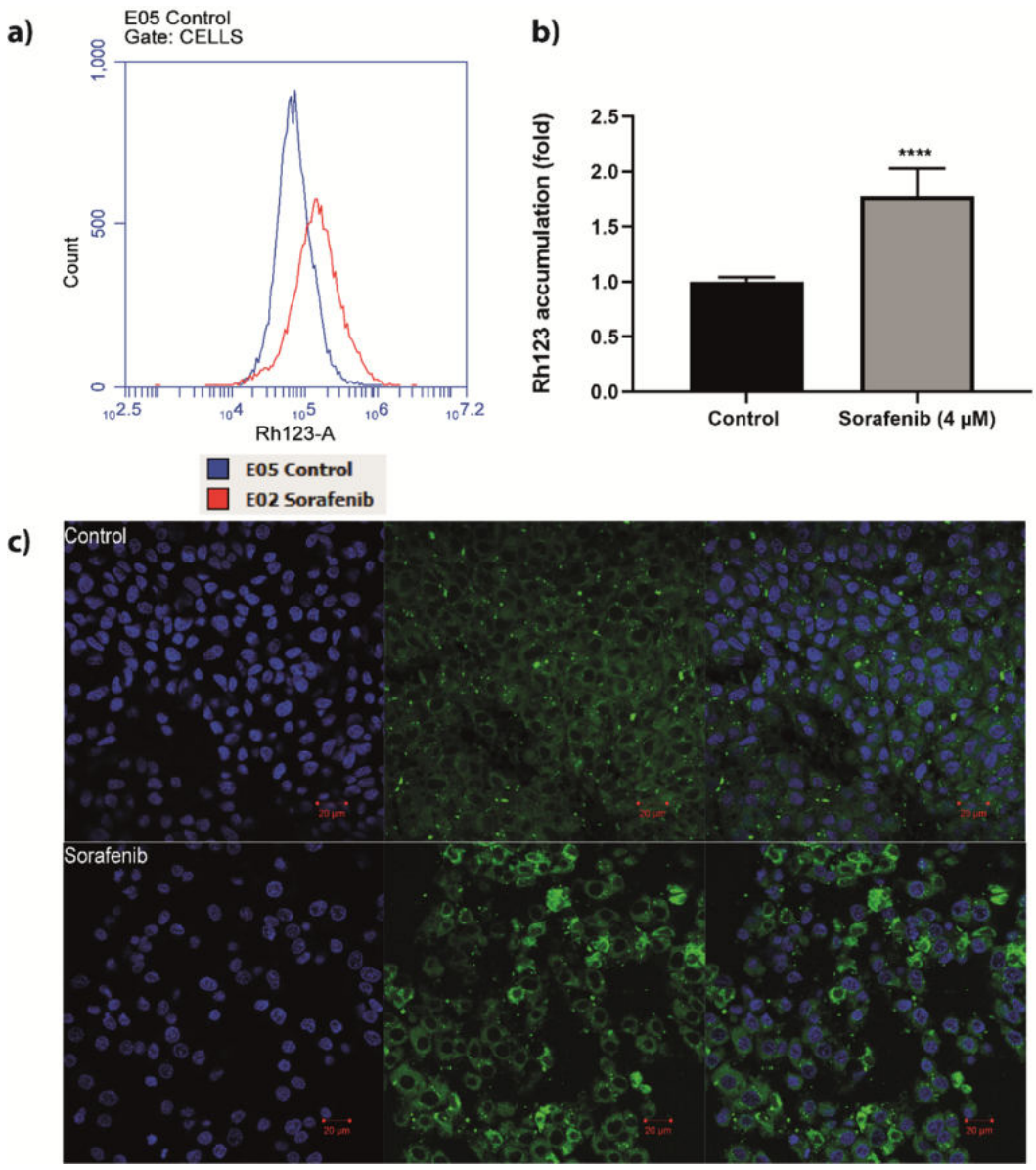

Fig. 3. Rh123 accumulation assays. (a) Histogram demonstrating the Rh123 fluorescence of control and sorafenib treated groups measured in FL-1 channel. (b) Bar graph showing fold change in Rh123 accumulation. Student's t-test was used to find significance. ****$p < 0.0001$ vs control group. (c) Confocal laser scanning microscopy of the control and sorafenib treated groups showing Rh123 accumulation (middle panel). Nuclei were counterstained with Hoechst 33258 (left panel). Merged images (right panel).

treatment for 72 hours, Vimentin and Snail1 expressions decreased significantly (0.42 fold, $p = 0.0009$ and 0.77 fold, $p = 0.0087$), while E-cadherin expression increased dramatically (4.36 fold, $p = 0.0010$) (Fig. 1).

Effects of Low-Dose Sorafenib Treatment on MDR-Related Gene Expression and Rh123 Accumulation

The association of sorafenib with multidrug resistance is a major obstacle for the treatment of HCC [18]. We explored whether low-dose sorafenib induces any changes in the mRNA levels of MDR-related genes and the accumulation of Rh123, which is effluxed by mainly P-gp and also to a lesser extent by MRP1. As shown in Fig. 2, while P-gp gene expression increased significantly (2.16 fold, $p = 0.0012$), no significant change was detected in the mRNA levels of MRP1 and BCRP (1.51 fold, $p = 0.0634$; 1.07 fold, $p = 0.7106$, respectively).

To examine if increased P-gp mRNA levels correspond to a change in cellular Rh123 accumulation, we measured and visualized Rh123 accumulation by flow cytometry (Figs. 3a and b) and confocal laser scanning microscopy (Fig. 3c), respectively. We demonstrated a 1.78 fold increase in Rh123 accumulation following sorafenib treatment for 72 hours ($p < 0.0001$). Similarly, sorafenib treated cells accumulated more Rh123 as evident by confocal laser scanning microscopy images.

DISCUSSION

Sorafenib is an approved agent in the treatment of advanced unresectable HCC. Sorafenib appears beneficial only approximately in 30% of patients. Unfortunately, this population develops drug resistance within 6 months [18]. Moreover, clinically significant toxicities develop in about 50% of the patients [3]. Adverse events such as diarrhea, weight loss, hypertension and hand and foot skin reactions generally occur within 2-6 weeks of the treatment [19]. Due to the high frequency and severity of the adverse effects, the patients generally undergo a dose reduction or drug discontinuation [3]. A recent study performed by Tak *et al*. [4] focused on progression-free survival, overall survival, duration of the treatment, cumulative dose, adverse effects, and drug discontinuation in HCC patients treated with sorafenib. The researchers recommended dose reduction to prolong survival and to improve the treatment efficiency with a higher cumulative dose and longer duration by increasing patient tolerance and adherence.

The aim of this study was to evaluate whether low-dose sorafenib treatment results in any EMT or MDR-related changes in HepG2 cells. Previously, our group evaluated the cytotoxicity of sorafenib in HepG2 cells and demonstrated approximately 75% cell proliferation following the treatment with 4 μM sorafenib [14]. In the same study, we also reported altered expression of the cancer stem cell (CSC) markers CD44, CD90, and CD133. The known link between the CSCs and the occurrence of EMT and MDR within tumors prompted us to examine the effects of low-dose and short-term sorafenib (4 μM) treatment on EMT and MDR by gene expression analysis and functional tests [13, 15].

EMT is a developmental process characterized by the loss of epithelial cell polarity, weakening of E-cadherin-related cell-cell adhesion and, the acquisition of mesenchymal markers such as Vimentin and N-cadherin through EMT-inducing transcription factors including Snail1 [6]. The clinical significance of EMT has been demonstrated in HCC. Reduced E-cadherin and overexpression of Snail1 were identified in 60.2% and 56.9% of primary HCC samples, respectively and the alterations of the two markers were shown to be associated with worse prognosis [20]. We found significantly lower mRNA levels of the mesenchymal markers Vimentin and Snail in response to low-dose sorafenib. On the other hand, the expression of the epithelial marker E-cadherin increased significantly, indicating a protective effect of low-dose sorafenib against EMT in gene expression level. Similarly, several studies reported that sorafenib inhibits EMT in a variety of experimental settings [21-23]. Moreover, sorafenib resistance is often associated with changes in the tumor microenvironment, and sorafenib-resistant cells were shown to exhibit EMT [24-26].

EMT and drug resistance with the overexpression of ABC transporters appear to correlate strongly [27-29]. A recent study reported that sorafenib-resistant HCC cells with the overexpression of Snail also exhibit increased levels of P-gp expression. Moreover,

the same study demonstrated the association of the reversal of resistance with the up-regulation of E-cadherin and simultaneous down-regulation of Snail, Vimentin and P-gp [24]. To investigate whether low-dose sorafenib alters MDR-related gene expression, we analyzed the mRNA levels of P-gp, MRP1 and BCRP. We obtained an about 2-fold increase in P-gp expression, while no change was detected in the expression levels of MRP-1 and BCRP. We also performed Rh123 accumulation analysis to measure the functionality of the efflux pumps. Rh123, a fluorescent substrate, is effluxed by mainly P-gp and also to a lesser extent by MRP1 [30]. Despite the increased P-gp expression, we showed an increase in Rh123 accumulation, corresponding to a lower efflux rate and a possible involvement of other molecular mechanisms. Rh123 was identified as a high-affinity substrate also for other transporters including for organic cation transporters 1 and 2 [31], and the increased P-gp expression might not be sufficient alone for an increased Rh123 efflux. Moreover, the interaction of sorafenib with ABC transporters is inconclusive. While sorafenib was suggested to have a moderate affinity for P-gp and negligible for BCRP [32], others propose that BCRP is rather important for sorafenib sensitivity [33, 34]. The measurement of Rh123 to determine the activity of ABC transporters is a limitation of this study, as the fluorescent molecule is also a high-affinity substrate for organic cation transporters 1 and 2. Another limitation is the lack of protein expression assays.

Sorafenib dose reductions or discontinuations because of the intolerable adverse effects or acquired or primary drug resistance are common during HCC treatment. Currently, combination therapies involving sorafenib at lower doses are hotspots in research and are expected to provide beneficial clinical outcomes [35]. Several studies were performed to examine if the dose reductions compromise the treatment outcomes, and the researchers suggest dose modifications for maximizing patient adherence and outcomes [4, 36-38].

CONCLUSION

In the current study, we examined the effects of low-dose sorafenib, yielding 75% cell proliferation, on EMT and MDR in HepG2 cells. We obtained an increase in epithelial marker E-Cadherin and downregulation of mesenchymal markers Vimentin and Snail1, indicating a protective effect of sorafenib against EMT. Moreover, we demonstrated upregulated P-gp expression, yet this change in mRNA levels did not result in a reduction in Rh123 accumulation in the cells. Other mechanisms are possibly involved and require further investigation. Understanding the EMT and MDR-related changes might contribute to lower sorafenib dose and enlighten new treatment strategies to overcome drug resistance.

Authors' Contribution

Study Conception: YDÇ, ZGÖ; Study Design: YDÇ, ZGÖ; Supervision: YDÇ, ZGÖ; Funding: YDÇ; Materials: YDÇ; Data Collection and/or Processing: YDÇ, ZA, GD; Statistical Analysis and/or Data Interpretation: YDÇ, RGA, ZGÖ; Literature Review: YDÇ, ZA, GD, ZGÖ; Manuscript Preparation: YDÇ, ZGÖ and Critical Review: YDÇ, ZA, GD, RGA, ZGÖ.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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