Translational Oncology
www.transonc.com

Volume 7 Number 6 December 2014 pp. 702–711 **702**

# Sequential Salinomycin Treatment Results in Resistance Formation through Clonal Selection of Epithelial-Like Tumor Cells[1,2]

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## Abstract

Acquiring therapy resistance is one of the major obstacles in the treatment of patients with cancer. The discovery of the cancer stem cell (CSC)–specific drug salinomycin raised hope for improved treatment options by targeting therapy-refractory CSCs and mesenchymal cancer cells. However, the occurrence of an acquired salinomycin resistance in tumor cells remains elusive. To study the formation of salinomycin resistance, mesenchymal breast cancer cells were sequentially treated with salinomycin in an *in vitro* cell culture assay, and the resulting differences in gene expression and salinomycin susceptibility were analyzed. We demonstrated that long-term salinomycin treatment of mesenchymal cancer cells resulted in salinomycin-resistant cells with elevated levels of epithelial markers, such as E-cadherin and miR-200c, a decreased migratory capability, and a higher susceptibility to the classic chemotherapeutic drug doxorubicin. The formation of salinomycin resistance through the acquisition of epithelial traits was further validated by inducing mesenchymal-epithelial transition through an overexpression of miR-200c. The transition from a mesenchymal to a more epithelial-like phenotype of salinomycin-treated tumor cells was moreover confirmed *in vivo*, using syngeneic and, for the first time, transgenic mouse tumor models. These results suggest that the acquisition of salinomycin resistance through the clonal selection of epithelial-like cancer cells could become exploited for improved cancer therapies by antagonizing the tumor-progressive effects of epithelial-mesenchymal transition.

*Translational Oncology (2014) 7, 702–711*

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[1] This article refers to supplementary materials, which are designated by Figures S1 to S4 and are available online at www.transonc.com.

[2] This work was supported by the German Research Foundation (SFB1032; project B4). A.H. was supported by a doctoral fellowship from the Islamic Development Bank (28/IND/P32). V.K.U. was supported by a grant from the Deutsche Krebshilfe Foundation (109081). Conflict of interest: The authors declare no conflict of interest.
Received 7 June 2014; Revised 5 September 2014; Accepted 11 September 2014


1936-5233/14
http://dx.doi.org/10.1016/j.tranon.2014.09.002

## Introduction

The acquisition of chemoresistance represents one of the major obstacles in cancer treatment. Albeit early detection methods improved and novel treatment options emerged, resistance formation to chemotherapeutics remains an enormous challenge for cancer therapy. In breast cancer, 30% of all patients with breast cancer suffer a relapse associated with metastasis and chemoresistance. The response rates for classic chemotherapy including anthracyclines and taxanes drop to only 20% to 30% on disease progression, even though the response rates for first-line chemotherapy are up to 70%. Several resistance mechanisms, such as the up-regulation of ATP binding cassette transporters and the overexpression and constitutive activity of growth factor receptors or certain other proteins and enzymes like β-tubulin III and thioredoxin [1–4], have been identified over the last years. In addition, recent findings on intratumoral heterogeneity [5–7] suggest that clonal evolution and plasticity are important drivers of the long-term resistance formation to chemotherapy. With regard to this, cancer stem cells (CSCs) became important targets of therapeutic approaches as they give rise to resistant subpopulations and thus are assumed to be one of the major causes of relapse and therapy resistance. Gupta et al. have found salinomycin to be a selective inhibitor of CSC, being 100-fold more effective than paclitaxel, a commonly used chemotherapeutic breast cancer drug [8]. Subsequent studies in a variety of different cancer types including breast, blood, lung, pancreas, and colon have revealed diverse mechanisms of salinomycin action against CSC resulting in apoptosis and cell death [9–12]. Interestingly, in several recent studies, salinomycin has been reported to induce apoptosis in cisplatin-resistant cancer cells [13–15], to overcome ATP binding cassette transporter–mediated drug resistance in leukemia cells [16], and to sensitize cancer cells to different chemotherapeutic drugs [17,18], hence circumventing the resistance to classic chemotherapy. Therefore, treatment with salinomycin in addition to classic cancer therapy could greatly improve the prognosis of patients with cancer. Several case studies applying salinomycin in a clinical setting revealed beneficial effects in patients with exhausted therapy options [9]. Because of these promising results, it is of great interest to investigate resistance formation to salinomycin treatment as the occurrence and the potential underlying mechanisms of an acquired salinomycin resistance in cancer cells remain elusive.

In this study, we sought to investigate the resistance formation to long-term sequential salinomycin treatment in an *in vitro* cell culture assay. We found that repeated salinomycin treatment resulted in a clonal selection of cells displaying more epithelial traits and increased resistance to salinomycin. Of note, in syngeneic and transgenic mouse tumor models, we also observed the selection of epithelial-like cell clones.

## Materials and Methods

### *Reagents*

These primary antibodies against the following proteins were used: actin (I-19) (SC-1616; Santa Cruz Biotechnology, Dallas, Texas) for Western blot (WB); vimentin (D21H3) XP (#5741; Cell Signaling Technology, Boston, Massachusetts) for WB and *in vivo* immunohistochemistry (IHC)/immunofluorescence (IF); E-cadherin (24E10) (#3195; Cell Signaling Technology) for WB and *in vivo* IHC/IF; E-cadherin (DECMA-1) (ab11512; Abcam, Cambridge, UK) for *in vitro* IF; vimentin (V9) (SC-6260, Santa Cruz Biotechnology) for *in vitro* IF. Salinomycin (S6201) was obtained from Sigma-Aldrich, Munich, Germany.

### *Cell Culture*

The breast (BT-474, MCF-7, MDA-MB-231, MDA-MB-436, and 4T1) and lung (NCI-H1299 and Lewis lung carcinoma) cancer cell lines were cultivated according to the supplier's instructions (ATCC).

### *Transfections*

For miR-200c overexpression experiments, cells were transfected with either Pre-miR miRNA Precursor of hsa-miR-200c (pre-miR-200c; Ambion) or Pre-miR miRNA Negative Control (control; Ambion) using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol.

### *Quantitative Reverse Transcription–Polymerase Chain Reaction*

Total RNA was isolated with the miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. miRNA or mRNA was reversely transcribed and subjected to quantitative reverse transcription–polymerase chain reaction (RT-PCR) as described previously [19]. All experiments were done in triplicate, and the following primers and hydrolysis probes (Roche, Penzberg, Germany) were used: E-cadherin (hsa), UPL Probe #35, left primer: CCCGGGACAACGTTTATTAC, right primer: GCTGGCTCAAGTCAAAGTCC; vimentin (hsa): UPL Probe #56, left primer: GTTTCCCCTAAACCGCTAGG, right primer: AGCGAGAGTGGCAGAGGA; zeb2 (hsa): UPL Probe #68, left primer: AAGCCAGGGACAGATCAGC, right primer: CCACACTCTGTGCATTTGAACT; Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) (hsa), UPL Probe #60, left primer: AGCCACATCGCTCAGACAC, right primer: GCCCAATACGACCAAATCC; E-cadherin (mmu): UPL Probe #18, left primer: ATCCTCGCCCTGCTGATT, right primer: ACCACCGTTCTCCTCCGTA; vimentin (mmu): UPL Probe #79, left primer: TGCGCCAGCAGTATGAAA, right primer: GCCTCAGAGAGGTCAGCAAA; zeb2 (mmu): UPL Probe #42, left primer: CCAGAGGAAACAAGGATTTCAG, right primer: AGGCCTGACATGTAGTCTTGTG; GAPDH (mmu): Universal ProbeLibrary Mouse GAPD Gene Assay (Roche); miR-200c stem loop primer (hsa and mmu): GTTGGCTCTGGTGC-AGGGTCC GAGGTATTCGCACCAGAGCCAACTCCATC; miR-200c forward primer: GCGTAATACTGCCGGGTAAT; universal reverse primer: GTGCAGGGTCCGAGGT.

### *Cell Lysis and Immunoblot Analysis*

WB experiments were performed as described previously [19] using the respective antibodies from the Reagents section.

### *Cell Viability Assay*

For cytotoxicity experiments, cells were seeded on 96-well plates at a density of 5000 cells per well. After 24 hours, cells were incubated with the respective drugs for 72 hours unless otherwise indicated. Subsequently, the CellTiter-Glo (Promega, Mannheim, Germany) assay was performed according to the manufacturer's protocol. Cell viability was normalized to the respective mock-treated control cells and presented as percent of control. Half maximal inhibitory concentration ($IC_{50}$) values for salinomycin were obtained from several different drug concentrations using GraphPad Prism software for analysis. All experiments were done in triplicate.

### *Long-Term In Vitro Salinomycin Treatment*

MDA-MB-436 cells received a long-term pulsed salinomycin treatment similarly as it has been described previously [19]. Briefly, MDA-MB-436 cells were treated with 50 nM salinomycin for