

Original Article

Research on mechanism of aramine for inhabitation of lung colonization of breast cancer

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Abstract: In this paper, the expression of Na_v1.5 voltage-gated sodium channels in breast tumors and the relationship between their expression level and metastatic occurrence were investigated. The aramine could inhibit Na_v1.5 currents in breast cancer cells and reduce Na_v1.5-related cancer cell invasiveness in vitro. The results demonstrated the importance of Na_v1.5 in the metastatic colonization and small molecules aramine interfering with Na_v activity, indicating a potential powerful pharmacological tool to inhibit metastatic development.

Keywords: Aramine, Na_v1.5, inhabitation

Introduction

Within all types of cancers in women, breast cancer nowadays becomes the primary cause of death, which usually attribute to the metastases. Up to now, there is rare efficient treatment to inhibit the development of metastases, because of the ability of cancer cells to degrade and migrate via extracellular matrices (ECM) [1]. As such typical characteristic for excitable cells, voltage-gated sodium channels (Na_v) are of great importance for action potential initiation [2]. However, in unexcitable epithelial cancer cells, such as breast [3], lung [4], ovarian [5], prostate [6], colon cancer [7], etc., different isoforms of Na_v have been investigated. It should be noticed that the isoforms of Na_v1.5 would be abnormally expressed in breast cancer biopsies rather than in normal mammary tissues, whose expression level is strongly related to the lymph node invasion, metastases development, as well as the reduction of patients' survival [8, 9]. A neonatal splice variant would be expressed in cancer cells, showing a 7-amino acid substitution in the segments S3 and S4 of the domain I (DI-S3-S4) of the protein [8, 10]. The adult variant with particular pharmacology was proposed to be metastatic marker. At the plasma membrane of highly invasive breast cancer cells, Na_v1.5 usually plays a role of maintaining pro-invasive phenotype via

mesenchymal migration [3, 11]. Na_v1.5 activity could not only control Src kinase activity, cortactin phosphorylation (Y421), and the subsequent polymerization of actin filaments, but also increase the activity of the Na⁺/H⁺ exchanger type 1 (NHE-1), enhancing the protons efflux and the proteolytic activity of extracellular released acidic cysteine cathepsins B and S. [1, 12] Despite the complete mechanism for Na_v1.5 activity is still unexplored not so far, these results indicated that both the invadopodial activity for breast cancer cells and the surrounding ECM invasion could be promoted by Na_v1.5 [13]. Its activity could be influenced by various molecules [3, 10]. For example, cancer cell invasiveness in vitro could be reduced by tetrodotoxin, while veratridine is helpful to the enhancement of ECM invasion. Aramine is a peripheral pressure boost drug that can excite α adrenergic receptor. In clinic, Aramine could be applied for the treatment of various early shock and the hypotension induced by surgical or spinal anesthesia. Nevertheless, there is rare contribution devoted to the importance of Na_v1.5 expression for the Aramine pharmacological inhibition on the metastatic colonization.

In this work, the expression of Na_v1.5 in human breast cancer cells and the pharmacological inhibition of Aramine for reduction of cancer cell invasiveness were investigated.

Materials and methods

Reagents

All drugs and chemicals were purchased from Sigma-Aldrich. Aramine was prepared in phosphate buffer saline (PBS).

Cell culture and cell lines

MDA-MB-231-Luc human breast cancer cells with stably luciferase gene expression were cultured in DMEM with 5% FCS at 37°C in a humidified 5% CO₂ incubator. A lentivirus vector encoding a short hairpin RNA (shRNA) specifically targeting human SCN5A transcripts were constructed according to the protocol in previous literature. The sequence encoding shNa_v1.5 was acquired by filling two partially complementary primers via DNA polymerase: 5'-GGATC-CCCAAGGCACAAGTGC GTGCGCAATTCAAGAGA-3' and 5'-AAGCTTAAAAAAGGCACAAGTGC GTGCGCAATCTCTTGAA-3'. Another lentivirus vector with untargeted shRNA (pLenti-shCTL) expression was also constructed by primers of 5'-GGATCCCCGCCGACCAATTACGCGCCGTTCAAGAGACG-3' and 5'-AAGCTTAAAAAGCCGACCAATTACGCGCCGCTCTTGAACG-3'. The shCTL and shNa_v1.5 cell lines with similar luciferase activity *in vitro* were obtained.

RNA extraction, reverse transcription, and real-time qPCR

Total RNA was extracted from MDA-MB-231 cells on a RNA Isolation. The purity and yield of RNA were examined by spectrophotometry, and the samples with A260/A280 ratio > 1.6 were picked out for downstream experiments. The total RNA was then reverse-transcribed and real time PCR was performed according to the previous literature [1]. For Na_v1.5, the sequences (expected sizes) involved in Primers were forward 5'-CACGCGTTCACTTCTTC-3' and reverse 5'-CATCAGCCAGCTTCTTACA-3' (208 bp); while for HPRT1, those were forward 5'-TTGCTGACCTGCTGGATTAC-3' and reverse 5'-TATG-TCCCCTGTTGACTGGT-3' (119 bp).

Cellular electrophysiology

In the whole-cell configuration, the sodium currents were recorded using borosilicate glass patch pipettes with resistance of 4-6 MΩ on an Axopatch 200B amplifier (Axon Instrument, USA) under voltage-clamp mode. The external sodium solution with pH value of 7.4 contained

140 mm NaCl, 4 mm KCl, 1 mm MgCl₂, 2 mm CaCl₂, 11.1 mm D-Glucose, and 10 mm HEPES; While The intra-pipette solution with pH value of 7.2 contained 15 mm NaCl, 130 mm KCl, 1 mm MgCl₂, 0.37 mm CaCl₂, 1 mm Mg-ATP, 1 mm EGTA, and 10 mm HEPES.

Cell viability

The cells were seeded in the wells (4 × 10⁴ cells per well in a 24-well plate) for a 5-day growing. The culture media with different Aramine concentration were changed every day. The viable cell number was counted by method of the tetrazolium salt assay, and then normalised to the control condition.

In vitro invasion assays

Cell invasiveness was examined using culture inserts with 8-μm pore size filters, according to the previous literature.

Epifluorescence imaging

Firstly, the cells were cultured on cover slips for 24 h with a Matrigel™-composed matrix coating with 25 fluorogenic substrate of gelatinases. Then, they were washed in PBS and fixed by ice-cold paraformaldehyde in PBS. The phalloidin-AlexaFluor594 was applied for staining. Epifluorescence microscopy was performed under a Nikon TI-S microscope.

Statistical analyses

All statistical analyses were performed using SPSS Statistical Package. The data were presented as mean ± standard deviation (SD). Wilcoxon test was applied for the comparison of the voltages and the availability-voltage relationships under different conditions. Mann-Whitney test was used to compare the data of mRNA expression, peak I_{Na}, as well as cell circularity index and Matrix-Focalized-degradation activity index. The cell invasiveness analyzed by Kruskal-wallis analysis followed by a Dunn's test. The *P* values are indicated as: * means *P* < 0.05, ** for *P* < 0.01, *** and for *P* < 0.001, and they were considered as statistically significant, while NS means not statistically different.

Results and discussion

The MDA-MB-231-Luc cells with stable expressing of null-target small hairpin RNA (shCTL)

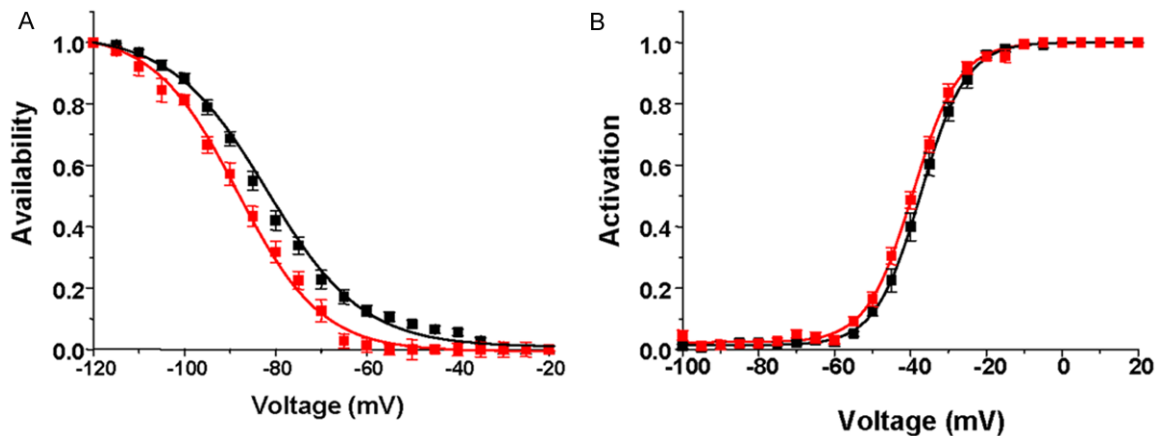
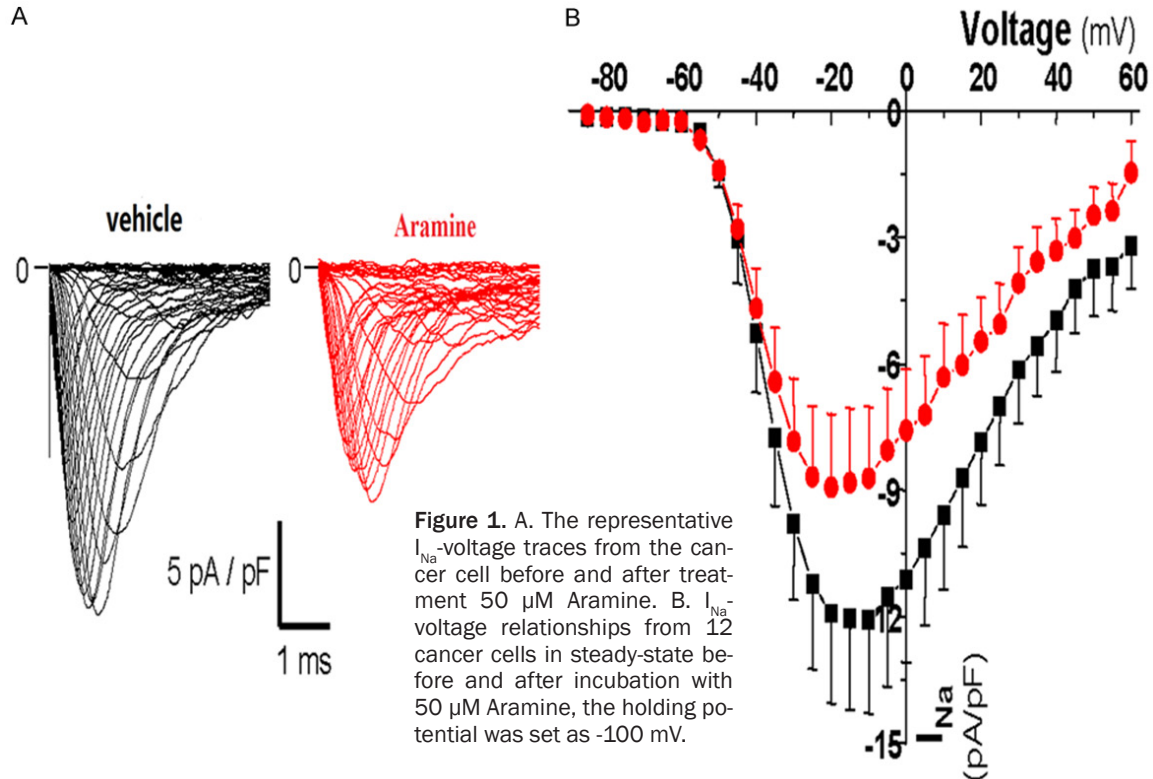


Figure 2. A. Availability-voltage relationships from vehicle group (black) and within 50 μ M Aramine (red). B. Activation-voltage relationships from vehicle group (black) and within 50 μ M Aramine (red).

were applied for sodium currents (I_{Na}) study by a voltage-clamp mode with the whole-cell configuration of the patch clamp technique. **Figure 1** shows there were presentative I_{Na} -voltage traces from the cancer cell before and after the treatment of 50 μ M Aramine, as well as the I_{Na} -voltage relationships between before and after incubation with 50 μ M Aramine from 12 cancer cells in steady-state, under condition of -100 mV holding potential. In **Figure 1B**, it can be

seen a statistical difference in the range of -35~+40 mV. The activation threshold was at about -60 mV and the maximal current was -12.1 ± 2.2 pA/pF located at -10 mV. **Figure 2** shows the availability-voltage (A) and activation-voltage (B) relationships between the vehicle group (black) and within 50 μ M Aramine (red), and significant leftward shifts in presence of Aramine could be observed both in availability-voltage and activation-voltage relationships.

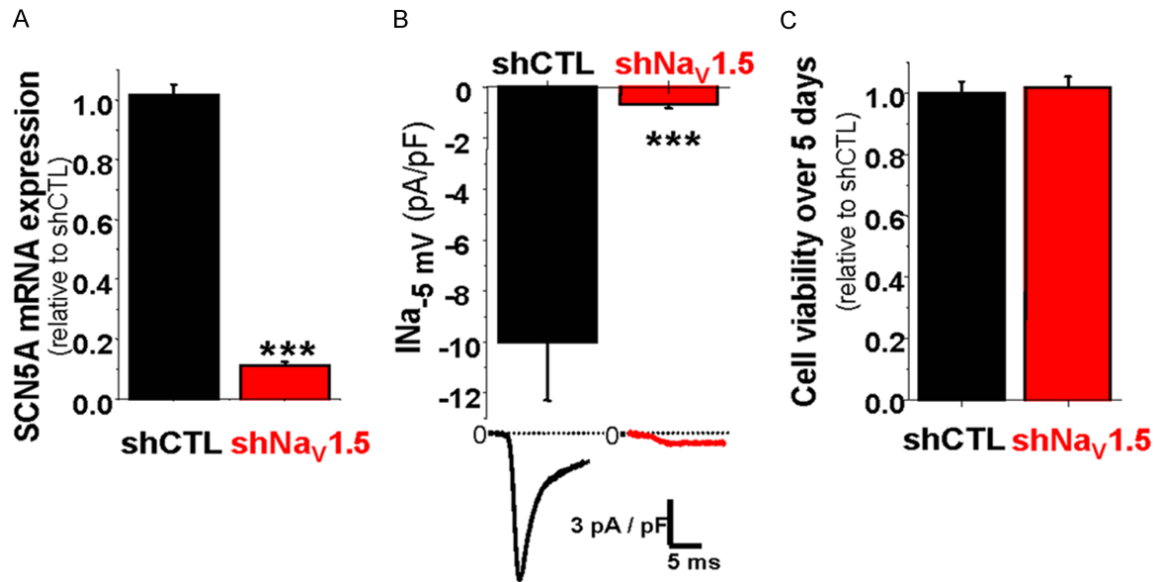


Figure 3. A. The real-time qPCR for SCN5A mRNA expression in 10 shCTL and 10 shNa_v1.5 cells. B. The I_{Na} peaks from 23 shCTL cells and 20 shNa_v1.5 cells under a depolarization from -100~-5 mV. The representative currents were also shown under the histogram. C. The viabilities of shCTL and shNa_v1.5 cells over 5 days, expressed relative to the shCTL cell line.

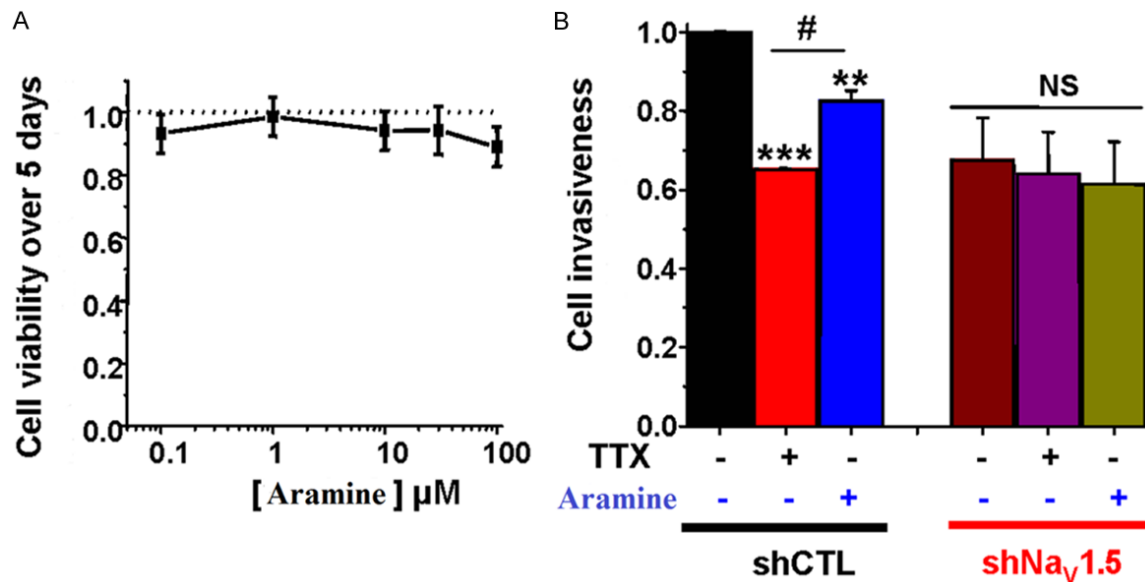


Figure 4. A. The viability of shCTL Cell over 5 days with increasing Aramine concentration (0.1~100 μM), expressed relative to the control vehicle group. B. Effect of 30 μM tetrodotoxin (TTX) or Aramine on invasiveness of shCTL and shNa_v1.5 cancer cell.

The maximal amplitude was significantly reduced to -8.7 ± 1.7 pA/pF by present of Aramine, relating to the above leftward shift. The half-inactivation voltage and half-activation voltage were shifted from -84.1 ± 1.4 and -37.1 ± 1.0 mV to -90.3 ± 1.7 and -39.2 ± 0.6 mV, respectively. Therefore, the activity of the neonatal Na_v1.5

isoform was efficiently reduced, suggesting that Aramine has inhabitation effect on sodium current in human breast cancer cells.

The inhabitation of Aramine on Na_v1.5-mediated breast cancer cell invasiveness *in vitro* was also investigated. The cells stably express-

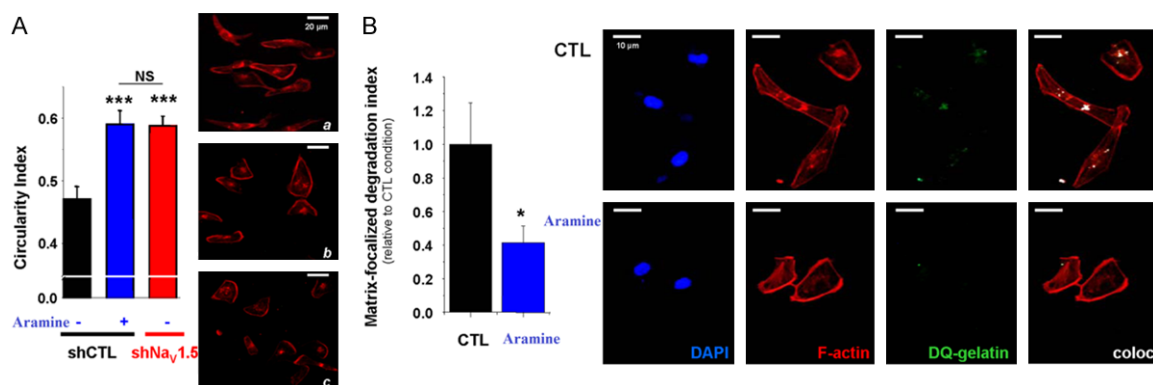


Figure 5. A. shCTL and shNa_v1.5 cells cultured for 24 h on a Matrigel™-composed matrix treated with or without 50 μM Aramine. F-actin cytoskeleton was stained with phalloidin-AlexaFluor594. B. shCTL cells cultured on a Matrigel™-composed matrix containing DQ-Gelatin with or without 50 μM Aramine.

ing a small hairpin RNA after lentiviral transduction selected targeting its expression (shNa_v1.5). The real-time qPCR for SCN5A mRNA expression in 10 shCTL and 10 shNa_v1.5 cells, as well as the I_{Na} peaks from 23 shCTL cells and 20 shNa_v1.5 cells under a depolarization from -100~5 mV were shown in **Figure 3A** and **3B**. A significant decrease of Na_v1.5 mRNA expression could be observed, leading to the complete disappearance of sodium currents in almost all cancer cells. However, it had no effect on viabilities of shCTL and shNa_v1.5 cells over 5 days, as shown in **Figure 3C**.

A possible cytotoxic effect of Aramine was evaluated for further assessing its effect on invasiveness reduction. **Figure 4A** shows the viability of shCTL Cell expressed relative to the control vehicle group over 5 days with increasing Aramine concentration (0.1~100 μM), indicating no effect on cell viability. Then the effect of 50 μM tetrodotoxin (TTX) or Aramine with Matrigel™-coated filters on invasiveness of shCTL and shNa_v1.5 cancer cells were investigated, as shown in **Figure 4B**.

For shCTL cells, cell invasiveness was reduced by 35±4% and 18±3% with TTX and Aramine, respectively. On the other hand, shNa_v1.5 cancer cells without Na_v1.5 expressing showed an invasiveness reduction of 33±10%. Neither TTX nor Aramine could further reduce cell invasiveness in shNa_v1.5 cells, indicating the specificity of Aramine for inhabitation of Na_v1.5-related invasion.

By maintaining the spindle-shape morphology and controlling the ECM proteolysis of MDA-MB-231 cells, the expression and activity of

Na_v1.5 could control the pro-invasive phenotype acquisition. It is found in **Figure 5A** that the circularity of shCTL cells was increased by Aramine, decreasing the pro-invasive morphology as a complete disappearance of Na_v1.5 expression. Moreover, the focal ECM degradative activity of shCTL cells was reduced Aramine by 58.6±10.0%, as shown in **Figure 5B**. The invadopodial activity was investigated by the fluorescence release from DQ-gelatin at focal sites of F-actin polymerization.

Conclusion

In this work, Aramine shows the effective pharmacological inhibition of Na_v1.5 channels for reducing metastatic colonization without toxic effect. The results demonstrate the importance of Na_v1.5 in the metastatic colonization and small molecules Aramine interfering with Na_v activity, indicating a potential powerful pharmacological tool to inhibit metastatic development.

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Disclosure of conflict of interest

None.

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