Scientific Report for COST STSM BM1406-36217

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Title: Changes in on fluxes across the plasma membrane of HMLE cells upon treatment with compound **613** using Microelectrode Flux estimation

Abstract

Based on the previous *in vitro* screening, we identified proprietary crown-ether compound **613** as a potential antitumor drug. Since crown ethers can act as K^+ ionophores, we hypothesized that anticancer activity towards breast tumor cells by **613** could be driven by disruption of potassium homeostasis.

Flow cytometric measurements using the cationic dye DiOC6(3) showed that compound **613** modulates membrane potential in breast cancer stem cell model (HMLE cells). It is extremely difficult to patch clamp HMLE cells, thus we used Microelectrode Flux estimation (MIFE), a non-invasive method based on measuring the concentration changes of ions close to the cells to examine ion fluxes. Specifically, we analysed fluxes of potassium and sodium ions through the plasma membrane of monolayers of HMLE cells and breast tumor cell lines MCF7 and SUM159. The results indicate that **613** in fact changes permeability of the plasma membrane of the examined cells for monovalent cations.

Aim of the study

To analyze modulation of fluxes of potassium and sodium ions induced by compound **613** in human breast cell lines using MIFE

Experimental design

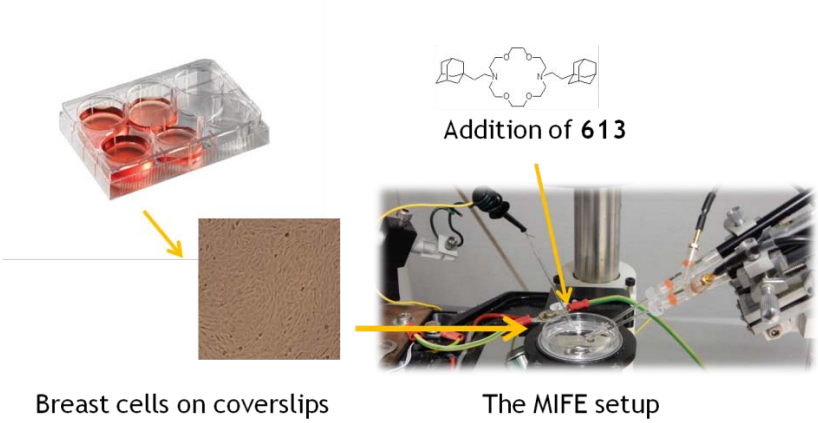

Figure 1. Human breast cell lines (breast cancer stem cell model cells and breast tumor cell lines) were grown in 6 well plates, on poly-lysin coated coverslipes. On the day of the experiment, cells were placed in the MIFE setup. We measured ion fluxes under various conditions, using K^+ and Na^+ selective electrodes.

Methods

1. Developed protocol for the preparation of human cells for MIFE

Human breast cell lines in culture- Cell Types:

HMLEpBp (transformed human mammary epithelia; control HMLE)

HMLETwist (HMLE overexpressing Twist Transcription Factor)

SUM159 (continuous breast cancer cell line)

MCF7 (continuous breast cancer cell line)

Maintenance: Grow cells in 75 cm2 flasks, split cells 2/3 times per week

Seeding for MIFE:

Use poly-lysin coated coverslips

Place each coverslip in one well of 6-wells plate or place them individually in petri dishes.

Pipette 400µl of cell suspension containing 0.5 x10^6 of cells directly onto coverslip and leave for 5-10 min. Add 1 ml of medium per well, carefully so concentrated cell suspension won't dilute with medium.

Next day (12-15 hr after) cells are ready for measurements. They should form confluent monolayer. If cells will grow for longer or shorter period of time, adjust the cell number required for seeding.

Prior to the measurements, wash cells with bathing solution.

2. CNSB developed protocol for Microelectrode based ion fluxes estimation (MIFE)

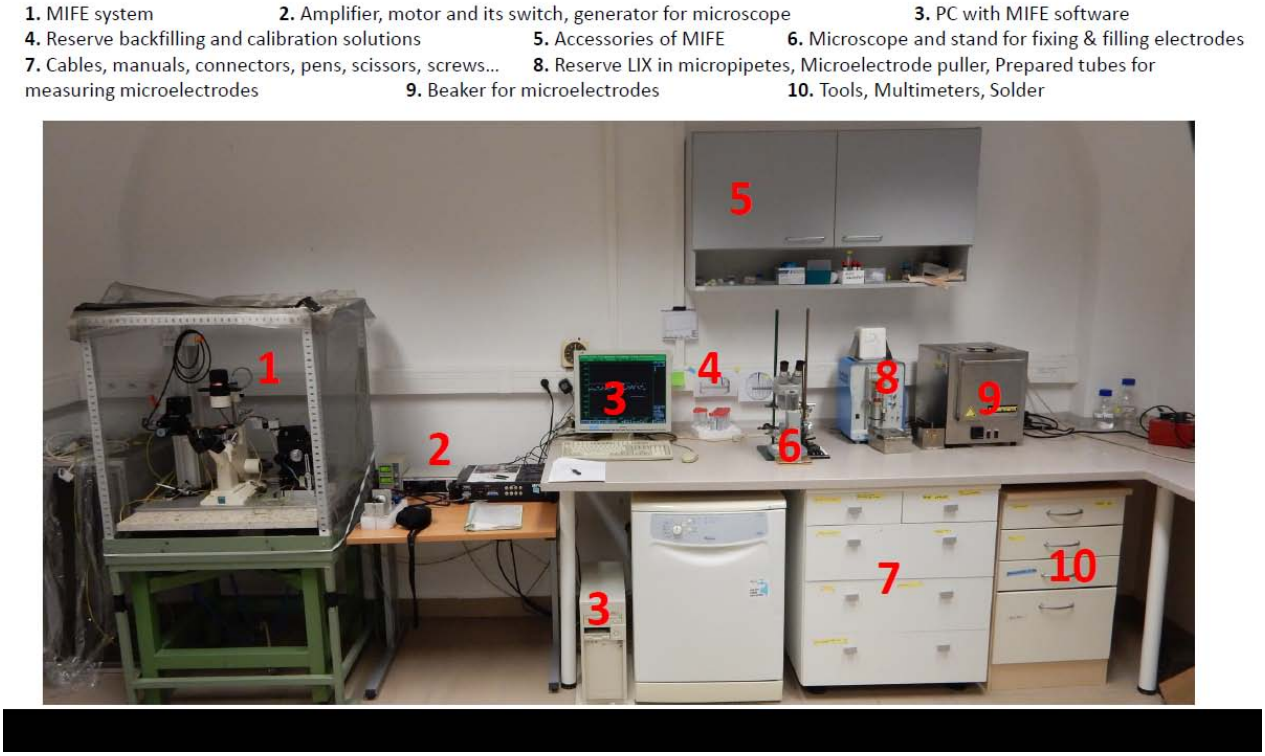

Figure 2. The MIFE setup and additional equipment at CNSB.

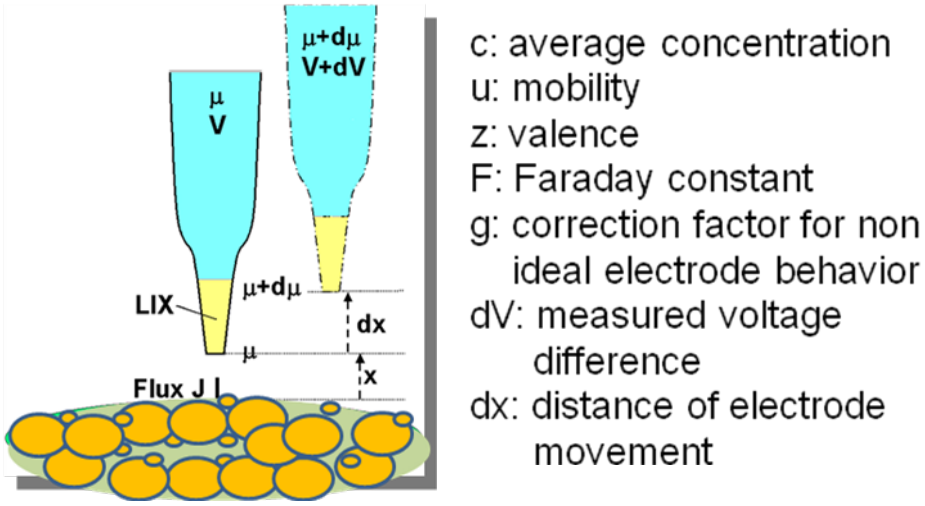

Figure 3. The MIFE principle: J = cuzFg(dVdx). Modified from: Shabala L, Ross T, McMeekin T, Shabala S. (2006) FEMS Microbiol Rev. 30:472-86. Non-invasive microelectrode ion flux measurements to study adaptive responses of microorganisms to the environment.

Results

1. Measurement of ion fluxes using MIFE

At first, we compared various bathing solution. We needed a solution that would be suitable for measurements of subtle changes in ion fluxes, and at the same time would allow cells to survive during longer measurements.

Example of bathing solution used:

HEPES/NaOH pH 7.45 10mM; ChCl 137 mM; $CaCl_2$ 1mM; $MgCl_2$ 0.5mM, Glucose 4g/l; KCl 1mM; NaCl 10mM

Also, we had to standardize endpoint, *e.g.* conditions and measured pattern that will point to total permeabilisation of cell's membrane and to cell's death. We chose steroidal saponine Digitonin.

Furthermore, we measured ion fluxes of different human cell lines induced using various compounds, until we obtained reproducibility. At the end, we measured changes in the ion fluxes induced by crown ether compound **613**.

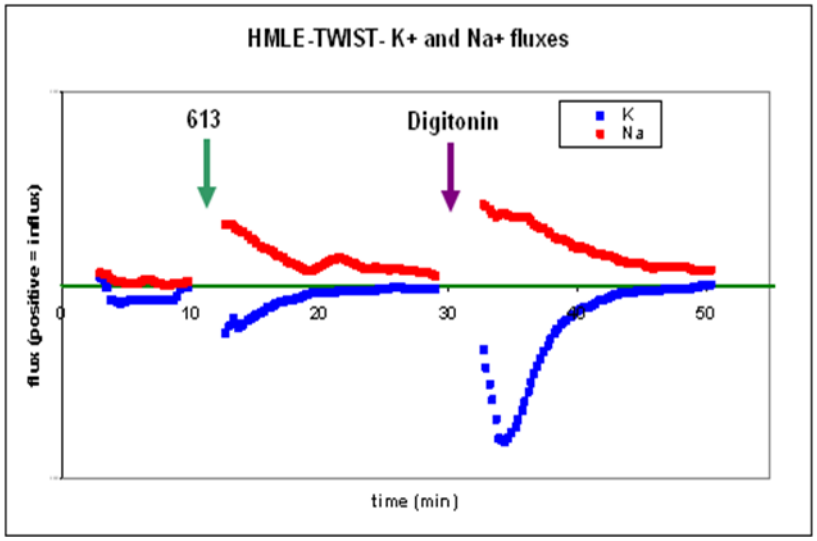

Figure 4. Representative plot of K^+ and Na^+ fluxes induced by **613** in HMLE cells. Compound induces K^+ efflux and Na^+ influx with HMLETwist cells. Permeabilization of the plasma membrane by Digitonin destroys the voltage gradient across the plasma membrane and thus leads to K^+ efflux and Na^+ influx.

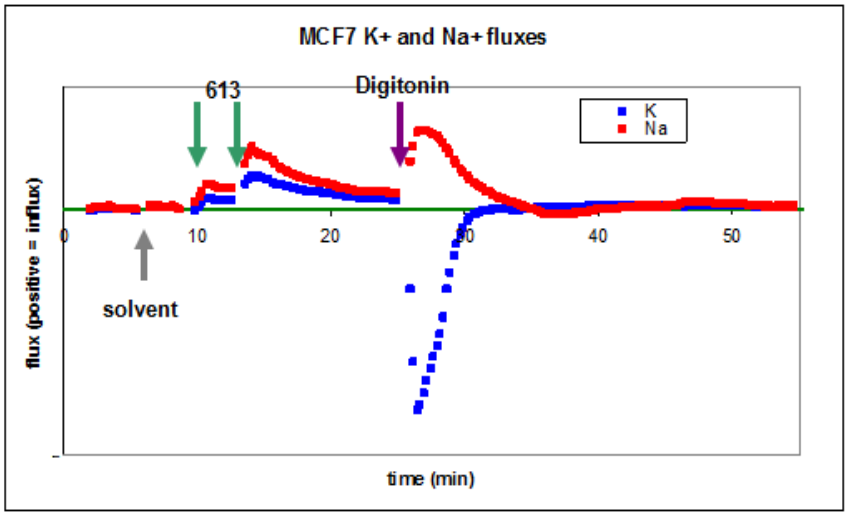

Figure 5. Representative plot of K^+ and Na^+ fluxes induced by **613** in MCF7 cells. Application of two different concentrations of **613** to MCF7 cells induces both K^+ and Na^+ influx, indicating a very negative membrane potential. Digitonin destroys the electrical gradient and leads to equilibration of internal and external ion concentrations. Solvent (DMSO) has no effect.

The electrode responses were not calibrated and thus fluxes cannot be quantified. This especially implies that magnitudes of Na^+ and K^+ fluxes are not necessarily comparable.

	"613"		Digitonin	
	K^+ flux	Na^+ flux	K^+ flux	Na^+ flux
SUM159	in	in	out	in
MCF7	in	in	out	in
HMLE-PbP	out (transient)	in	out	in
HMLE-TWIST	out	in	out	in

Bath solution contained 15 mM Na^+ and 1 mM K^+.

Table 1. Direction of K^+ and Na^+ fluxes after addition of **613** and subsequent permeabilization of cell membranes with Digitonin.

In general, our results indicate that under the conditions used SUM159 and MCF7 cells posses a very negative membrane potential (ca. -120 mV assuming internal [K^+] ~ 100 mM, [Na^+] ~ 5 mM). HMLEpBp and HMLETwist are less hyperpolarized.

Conclusions

MIFE is a suitable method for monitoring ion fluxes in human breast cell lines.

Compound **613** induces efflux of K^+ and influx of Na^+ in cancer stem cell model cells (HMLE).

In breast tumor cell lines MCF7 and SUM159 **613** induces influx of both ions.

Although directions of fluxes are in correlation with previously measured resting potential values, it seems that under our conditions SUM159 and MCF7 posses much more negative membrane potentials.

Additional measurements are required to quantify ion flux changes by MIFE after addition of **613.**

Acknowledgments This investigation was approved by COST as COST-STSM-BM1406-36217.

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