**Research article**

**Motility and proliferation of the triple negative breast cancer cell line MDA-MB 231 are selectively impeded by the ionophor salinomycin**

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**Abstract**

**Introduction** Tumour cells with the marker profile CD44+/CD24low/- have a high tumour-inducing potential. Salinomycin can specifically inhibit such cells. The effects of salinomycin on the viability and migration of triple negative breast cancer cells on triple negative breast cancer cells are to be investigated.

**Methods** MDA-MB 231 and redifferentiated subclone as control. The viability was determined with the MTT test, and the migration was determined using 24-hour videography.

**Results** Salinomycin leads to an inhibition of all migration parameters on MDA-MB 231. A highly significant correlation was shown between increase in salinomycin concentration and loss in cell viability, which was significantly less noticeable in the control group.

**Conclusion** In salinomycin there is a specific inhibition of MDA-MB 231. Since MDA-MB 231 has over 90% CD44+/CD24low/- positive cells, these are to be seen as a possible point of attack for salinomycin.

Key words: salinomycin, triple negative, cell migration, proliferation

**Introduction**

Breast cancer, with more than a million new cases per year worldwide, is one of the most common malignant diseases in women (1). Immunhistochemical criteria as well as analyses of gene expression allow a specific classification of this disease entity. This leads to the therapeutic use of anti-hormonal therapies, or a targeted antibody therapy with Trastuzumab in the case of overexpression of HER2 (2).

By definition, triple negative breast cancers have neither an expression of hormone receptors, nor an overexpression of HER2, such that anti-hormonal and targeted therapies are not promising (3).

In order to explain the formation of malignant tumours, the cancer stem cell hypothesis has moved into the focus of research. This was first postulated in 1997 by Bonnet and Dick with respect to acute myeloid leukaemia and in 2003 was transferred to breast cancers. According to this hypothesis, breast cancer cells with the surface profile CD44+/CD24low/- have a considerably higher tumour-inducing potential (4,5). Triple negative breast cancers contain a high proportion of the above-mentioned cell fraction (6).

Salinomycin is a carboxylic ionophor with an antibiotic effect which can specifically transport monovalent positive ions, with a preference for potassium ions, across biological membranes. It has already been used for decades in poultry farming to prevent coccidiosis and as a feed additive in pig and cow breeding to stimulate growth (7,8). In 2009 it was possible to demonstrate a specific cytotoxic effect on breast cancer cells with the surface marker profile CD44+/CD24low/- (9).

The breast cancer cell line MDA-MB 231 is a triple negative cell line with a mesenchymal phenotype and over 90% expression of CD44+/CD24low/- cells (10). Taking into consideration the results of Gupta, a significant influence of salinomycin is therefore also to be expected on cells of the MDA-MB 231 line, which would allow conclusions to be drawn on the effectiveness of this substance as an additional therapeutic option in triple negative breast cancers.

**Materials and methods**

**Cell line**

The experiments were carried out on the established triple negative breast cancer cell line MDA-MB 231. As a comparison group there was a redifferentiated subclone in which a CK-18 expression vector had previously been incorporated by means of transfection.

**Videography**

For a videography sequence, the cells were seeded into six Petri dishes of a 35x10 mm format, at a density of 16,000 cells per dish. The influence of salinomycin on the motility of the two cell lines was investigated at the concentrations 5x10-7, 10-6, 5x10-6, 10-5 and 5x10-5 mol/l. The videography apparatus was constructed on an inverted microscope connected with a digital camera. Instead of the XY table, a climatised chamber was attached in which, inside a six-hole tray, a maximum of 6 individual Petri dishes measuring 35x10 mm could be laid. The chamber was sealed gas-tight and the temperature was stabilised at 37°C. The chamber could be positioned freely along all axes using 3 linear motors. The control software program “MicroControl” made it possible to choose any number of fields of vision, which could be stopped at and photographed at freely selectable time intervals. The motility of the cells was observed over 24 hrs. Three fields of vision with approx. 20-30 cells each were chosen and photographed at intervals of 15 minutes. The image data from each field of vision were evaluated using the program “sci Taxis”. The following parameters were evaluated: migration speed (V) (µm/min), Euclidean distance (ED) (µm/24h) and accumulative distance (AD) (µm/24h).

**Data analysis**

The data gained were analysed with the program “Chemotaxis and Migration Tool” provided by Ibidi. The statistical analysis was carried out using the t-test for unpaired samples.

**MTT assay**

For the MTT assay the cells of the relevant cell line were seeded at a density of 5000 cells/well into a 96-well tray. Tests were made with salinomycin concentrations of 10-7, 5x10-7, 10-6, 5x10-6, 10-5, 1.25x10-5, 2.5x10-5, 5x10-5, 7.5x10-5, 10-4 and 5x10-4 mol/l. The cell viability was determined after 24 hrs and 72 hrs. The photometric measurement was carried out with a maximum absorption of 570nm.

**Analysis of Data**

After photometric measurement, the data gained were normalised and converted into %. The statistical evaluation was carried out using the t-test for unpaired samples and regression analysis.

**Results**

**Migration Speed (V)**

In salinomycin, with MDA-MB 231, a statistically highly significant (p<0.0001) loss in migration speed was visible as early as with a minimum concentration of 5x10-7 mol/l.

However, with the cells transfected with CK 18 no effect could be shown (Figure 1a).

**Accumulative Distance (AD)**

Under the influence of salinomycin, in MDA-MB 231 there was a statistically highly significant decline in AD (p<0.0001). Here, too, no inhibiting effect could be shown on the control group (Figure 1b).

**Euclidean Distance (ED)**

Unlike the migration speed and accumulative distance, among the MDA-MB 231 cells a statistically significant decline (p<0.05) in this parameter could only be shown starting with a salinomycin concentration of 10-5 mol/l upwards. No inhibiting effect was shown on the control group (Figure 1c).

**Cell viability**

For the direct comparison of the viability of cells of the control group transfected with

CK 18, the calculation of IC 50 was carried out with the help of regression analysis. Here after 24 hrs a salinomycin concentration 60 times higher was required to attain the IC 50 with the cells transfected with CK18 than with MDA-MB 231. After 72 hrs the salinomycin concentration required to reach IC 50 with the cells transfected with CK 18 was still 10 times higher than with the MDA-MB 231 cells.

**Discussion**

The inhibiting effect of salinomycin on tumour cells with stem cell properties has been known since the work of Gupta et al., and since then has been shown in further tumour entities such as osteosarcoma (11) or endometrial cancer (12). It is therefore assumed with respect to salinomycin that it has a specific effect on tumour cells with stem cell properties. MDA-MB 231 is a triple negative cell line with poor differentiation and with a mesenchymal phenotype, along with a high expression of vimentin and the associated high invasiveness (13). The tumour stem cell markers of breast cancer, according to Al-Hajj et al., consist of a combination of the surface markers CD44+/CD24low/- (14). Admittedly, according to Fillmore and Kuperwasser, MDA-MB 231 cells contain the above-mentioned surface marker profile in a proportion of more than 90%, a finding which was also confirmed by investigations by Borgna et al., but this alone does not yet entail a higher tumour-inducing effect (15,16). However, higher tumour induction was possible through a cell population with the marker profile CD44+/CD24low/- ESA+, yet this is a fraction which only constitutes approx. 2% of the cell mass (15).

The aim of this work was therefore to investigate the question to what degree cell viability and migration of the triple-negative breast cancer cell line MDA-MB 231 are influenced by the effect of salinomycin. A comparison group for this was provided by the redifferentiated subclone transfected with CK18. In migration tests on various cell lines, including MDA-MB 231, Kopp et al. were able to demonstrate an inhibition of migration ability in salinomycin in the Boyden chamber assay or in the scratch assay. Based on the results it was assumed that as well as tumour stem cells other tumour cells without the defined stem cell characteristics such as tumour induction could also be inhibited in their migration (16). The results found in this study confirm the assumptions of Kopp et al. Here videography is superior to the other two methods – migration in the Boyden chamber and the scratch assay – as it exclusively measures the migration potential of the cells investigated. In transwell experiments a cytokine gradient must be created so that cells can migrate through the membrane. What is measured is therefore inevitably always a mixture of migration and chemotaxis. In the scratch assay, the wound is not only closed by migrating cells, but also by a proliferation of the edges. Such side effects which falsify the migration values are not to be expected in the analyses through videography. Using 24-hr videography, in the salinomycin concentration range between 5x10-7 mol/l and 5x10-5 mol/l a statistically significant decline in all three migration parameters (V, AD and ED) could be demonstrated in MDA-MB 231. Similar results were shown with respect to the cell viability. For both cell lines (MDA-MB 231 and MDA-MN 231 (CK18)) a highly significant correlation could be demonstrated between increasing salinomycin concentration and drop in cell viability, a finding which is also supported by viability studies of An et al. (17). Additionally, by finding the IC 50 it was possible to find an increased tolerance to salinomycin in MDA-MB 231 CK18 in comparison to MDA-MB 231. In a direct comparison of the cell viability curve with the curves of migration parameters, the results additionally indicate that there are salinomycin concentration ranges respectively defined for MDA-MB 231 and MDA-MB 231 (CK18) in which the loss of viability is very low overall. Interestingly, in these concentration ranges there is also a stable curve for all migration parameters, such that it may possibly be assumed that the migration in the measured salinomycin concentrations is dependent on the cell viability. The fact that in the salinomycin concentration range investigated in this work between 5x10-7 mol/l and 5x10-5 mol/l no significant effect could be demonstrated on all parameters of the migration of the MDA-MB 231 (CK18) cell line may be due to the fact that the salinomycin concentration range investigated in the migration tests was precisely in the concentration range which only featured a low loss in viability. Possibly, for isolated observations of cell mobility even lower salinomycin concentrations would need to be selected.

According to Fillmore and Kuperwasser, the MDA-MB 231 cell line only has a 2% proportion of cells with stem cell properties. The results of this work therefore indicate that salinomycin also acts effectively on cells without stem cell properties.

The present work shows that that the specific inhibition by salinomycin of tumour cells with stem cell characteristics described by Gupta is also effective in breast cancers of the triple negative subtype. Ionophors such as salinomycin may therefore potentially be an option in the therapy of this tumour entity, which is difficult to access medicinally.

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