Influence of salinomycin treatment on division and movement of individual cancer cells cultured in normoxia or hypoxia evaluated with time-lapse digital holographic microscopy

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ABSTRACT
Most studies on new cancer drugs are based on population-derived data, where the absence of response of a small population may pass unnoticed. Thus, individual longitudinal tracking of cells is important for the future development of efficient cancer treatments. We have used digital holographic microscopy to track individual JIMT-1 human breast cancer cells and L929 mouse fibroblast cultivated in normoxia or hypoxia. In addition, JIMT-1 cells were treated with salinomycin, a cancer stem cell targeting compound. Three-day time-lapse movies were captured and individual cells were analysed with respect to cell division (cell cycle length) and cell movement. Comparing population-doubling time derived from population-based growth curves and individual cell cycle time data from time-lapse movies show that the former hide a sub-population of dividing cells. Salinomycin treatment increased the motility of cells; however, this motility did not result in an increased distant migration i.e. the cells increased their local movement. MCF-7 breast cancer cells showed similar motility behaviour as salinomycin-treated JIMT-1 cells. We suggest that combining features, such as motility and migration, can be used to distinguish cancer cells with mesenchymal (JIMT-1) and epithelial (MCF-7) features. The data clearly emphasize the importance of longitudinal cell tracking to understand the biology of individual cancer cells under different conditions.

Introduction
In cancer, the intra-tumour heterogeneity displayed as differences in e.g. cell morphology, gene expression, metabolism, proliferation including cell cycle dynamics, metastatic potential, and the tumour microenvironment have become more and more obvious. The impacts of this heterogeneity on cancer treatment outcome clearly needs further elucidation as these factors affect drug sensitivity. A sub-population of cells presumed to be part of the heterogeneity is cancer stem cells (CSCs). In the search for CSC specific drugs, using a high-throughput screening system, the ionophore salinomycin was found to efficiently target this population. Besides reducing the number of CSCs, salinomycin has been shown to reduce the migration of cells through inhibition of epithelial to mesenchymal transition (EMT) and by inducing mesenchymal to epithelial transition (MET). The EMT process is known to be a major cause of metastasis, making it an interesting drug-target to reduce the metastatic burden of patients.

It is well known that tumours have areas of both fixed and cyclic hypoxia. This is caused by the abnormal structure and function of the micro vessels supplying the tumour, resulting in increased diffusion distances between blood vessels and the tumour cells. Hypoxia causes a multitude of changes in gene expression, which contribute to the malignant process and development of tumour heterogeneity. CSCs have e.g. been shown to thrive in areas of tumour hypoxia. Thus, it is important to study individual cancer cell behaviour in both contexts of normoxia and hypoxia.

Most studies of effects of new drugs on proliferation and migration of cancer cells in culture are performed on a population level. However, studying cancer cells on a single cell level can give information that otherwise is hidden in population-based data. Differences in drug responses between cancer cells in a population can give an explanation to the outcome of treatment. By using live cell time-lapse imaging followed by analysis of individual cells, both time dependent interactions between cells and effects of drugs on individual cells can be monitored. This information can be used in developing and designing new target-directed drugs. Digital holographic microscopy is well suited for live cell imaging as no labelling or stains are necessary. As the illumination intensity is very low, there is no phototoxicity, allowing cells to be monitored and tracked over the course of several days. The images can be used for cell division and motility studies, as well as for cytotoxicity and cell morphology studies.

We have used digital holographic imaging to obtain time-lapse movies to monitor and track individual human breast cancer cells cultured in normoxia or hypoxia evaluated with time-lapse digital holographic microscopy.
cancer JIMT-1 and MCF-7 cells and mouse fibroblast L929 cells, cultured in normoxia (21% O₂) or hypoxia (1% O₂). In addition, the JIMT-1 cells were treated with salinomycin as we have extensive knowledge on a population-based level of how the treatment affects the CSC population. The data was used to create phylogenetic trees of cell divisions and to monitor the movement of individual cells. Our data shows that there is variability in cell behaviour, which indicates the need for individual lineage tracing experiments in order to increase our understanding of the heterogeneity of cancer cell populations and how this affects responses to different conditions including treatments.

**Results**

**Cell proliferation**

As the basis for our study, we established growth curves for the L929 and JIMT-1 cell lines in the conventional way by cell counting. The growth curves show that L929 cells were more sensitive to hypoxia than the JIMT-1 cells (Figs. 1A and 1B). The rate of cell proliferation estimated as population doubling time (Table 2) of L929 cells was lowered already during the first 24 h in hypoxia compared to normoxia and cell proliferation was inhibited totally when cells had been incubated in hypoxia for 48 h (i.e. between 72 to 96 h after seeding). The proliferation of JIMT-1 cells was similar in normoxia and hypoxia 24 to 72 h after seeding and thereafter proliferation decreased in hypoxia (compare population doubling times in Table 2). Salinomycin treatment reduced cell proliferation of JIMT-1 cells to a similar extent under both normoxic (Fig. 1C) and hypoxic (Fig. 1D) conditions.

**Individual cell cycle time**

The next step was to use time-lapse holographic movies to extract information regarding cell division of individual cells during the same time period as the population doubling times were calculated. Cell family trees were constructed (Fig. 2) and cells were categorized according to four different fates (Table 1). In Fig. 2, cell 1 shows the fate of a mother cell that was tracked from the first frame of the time-lapse, until the division into two daughter cells (Cells 1.1 and 1.2). The start of the cell cycle for the mother cell is unknown. Such cells are depicted as orange Xs in the bottom panels of Figs. 3, 6, 7, and 8. If the daughter cells divide later in the time-lapse, their entire cell cycle is known. Such cells are depicted with open circles in Figs. 3, 6, 7, and 8. Daughter cells that do not divide during the time-lapse are marked with red Xs (Figs. 3, 6, 7, and 8). Cell 2 shows the fate of a cell that was tracked from the start of the time-lapse and which did not divide throughout the 72 h observation period. Thus, this is a cell where there is no known start and end of the cell cycle. Such cells are marked with purple Xs (Figs. 3, 6, 7, and 8).

![Figure 1](image_url)

*Figure 1.* Growth curves for L929 and JIMT-1 cells cultivated under normoxic or hypoxic (1% O₂) conditions without or with treatment with 0.5 μM salinomycin. The cells were seeded on day 0. Twenty-four h after seeding (arrows in figures), the cells were subjected to the different incubation conditions shown in the figures. The cell number was determined by counting in a hemocytometer after detachment of cells with trypsin. Data represents mean ± SD for n = 3. *P < 0.05, **P < 0.005, ***P < 0.0005, Students T-test (unpaired).*
There are different reasons for an unknown end of the cell cycle, e.g. a very long cell cycle time, or the cell moved out of the frame during the time-lapse experiment, or the cell clumped together with other cells, which did not permit continued tracking. To exclude possible bias from the person performing the tracking, all tracked cells are included in the figures.

The x-axis label “Time-lapse (h)” in Figs. 3 and 4 equals time of treatment, i.e. time point 0 in Figs. 3 and 4, equals 24 h after seeding in the growth curves (Fig. 1).

The cell cycle time for individual L929 cells cultivated in normoxia did not vary much during the observation time (Fig. 3A, upper panel), shown by the almost horizontal regression line. For L929 cells cultivated in hypoxia (Fig. 3B, upper panel), the cell cycle time increased with culturing time as shown by the positive slope of the regression line. When comparing population doubling time and mean cell cycle time (Table 2) for L929 cells cultivated in normoxia or hypoxia, it is clear that the latter gives information about the presence of rapidly dividing cells that were not detected in the population-derived data. Table 2 also shows the percentage of cells that were still cycling during the last 24 h of the experiment. Thus, for L929 cells in hypoxia, the growth curve showed complete growth inhibition between 72 and 96 h after seeding while the tracking of individual cells showed that 3% of the tracked cells were still dividing although with prolonged cell cycle times.

A similar pattern for cell cycle times was found for JIMT-1 cells cultured in normoxia and hypoxia as described for L929 cells i.e. the cell cycle time for individual JIMT-1 cells cultivated in normoxia did not vary much during the observation time (Fig. 3C, upper panel, almost horizontal regression line) while it increased for cells cultured in hypoxia (Fig. 3D, upper panel) during the experimental period shown by the positive slope of the regression line. Comparing cell cycle times and population doubling times, it is apparent that the population-based growth curve is lacking information that can be found with individual cell tracking.

When JIMT-1 cells, cultivated in normoxia or hypoxia, were treated with salinomycin, the cell cycle time of dividing cells increased gradually during the experimental period (compare Figs. 3E and 3F including slopes, upper panels). Although the growth curves show a complete inhibition of cell proliferation after 48 and 24 h of treatment of cultures in normoxia and hypoxia, respectively, the analysis of individual cells show a small percentage of cells that were still proliferating (Table 2).

Cells with non-completed cell cycles (for both cell lines) are also included in Fig. 3, shown in the lower panels. The remaining Xs are cells that have not divided during the tracking time (red and purple Xs). Incubation in hypoxia and/or treatment with salinomycin resulted in an increased number of tracked JIMT-1 cells that did not divide during the time-lapse.

**Time dependent effect on cell division**

Figs. 4A-D show the time from start of the time-lapse until the first division of the tracked cells that divided i.e. the cells marked with yellow crosses in the lower panels of Fig. 3. All initially tracked L929 cells (Fig. 4A) and JIMT-1 (Fig. 4B) cells cultivated in normoxia eventually divided as shown by the number reaching 100%. Cultivation in hypoxia prevented about

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**Table 1.** Descriptions of symbols used in Figs. 2, 3, 4, 6, 7, and 8.

| Description of cell fate | Sample | Figure |
|--------------------------|--------|--------|
| ☓ Completed cell cycles. | 1.929 normoxia: ☓ | 3,6,7,8 |
| ☓ 1.929 hypoxia: ☓ | | |
| ☓ JIMT-1 normoxia: ☓ | | |
| ☓ JIMT-1 hypoxia: ☓ | | |
| ☓ JIMT-1 normoxia 0.5 μM salinomycin: ☓ | | |
| ☓ JIMT-1 hypoxia 0.5 μM salinomycin: ☓ | | |
| ☓ MCF-7 normoxia: ☓ | | |
| ☓ Cells where the start of the cell cycle is unknown, but the end is known. | | 3,4,6,7,8 |
| ☓ X X: Cells where the start of the cell cycle is unknown, but the finish is unknown due to end of tracking before division. | | 3,6,7,8 |
| ☓ Cells where neither start nor finish of cell cycle is known. | | 3,6,7,8 |
| ☓ /: Last division of each branch. | | 4 |
30% of the initially tracked L929 cells (Fig. 4A) and 20% of the initially tracked JIMT-1 cells (Fig. 4B) from dividing during the time-lapse (i.e. the percent divided cells reached 70%, and 80%, respectively). Salinomycin treatment of JIMT-1 cells cultivated in normoxia (Fig. 4C) or hypoxia (Fig. 4D) prevented about 20% of the initially tracked cells from dividing during the time of the time-lapse i.e. similar result as cultivation in hypoxia alone.

Figs. 4E-H show the time to the last division of each branch in each cell tree. Culturing cells in hypoxia (Fig. 4E, L929 cells and Fig. 4F, JIMT-1 cells) or treating with 0.5 μM salinomycin (Fig. 4G, JIMT-1 cells in normoxia and Fig. 4H, JIMT-1 cells in hypoxia) shifted the curves to the left, demonstrating a total inhibition of cell division in more trees at earlier time points than in respective controls. Here, salinomycin treatment had a higher impact than hypoxia compared to the effect on the first cell division where salinomycin treatment and hypoxia showed similar effects (compare Figs. 4D and H).

**Cell movement**

In addition to investigating cell division using the HoloMonitor M3 or M4, it is also possible to track cell movement in the acquired time-lapses using the software HStudio™. In Hstudio™, cell movement can be described by the parameters motility and migration (Fig. 5). Motility is defined as the total accumulated distance a cell has moved over the time of tracking. Migration is defined as the shortest distance from the starting point of the tracking to the point where the cell is located in each captured frame. For each individual cell, migration directness is calculated as the ratio between migration and motility for each frame. In average, for each cell, approximately 240–360 migration directness values were calculated, depending on the time of tracking. The average migration directness was then calculated based on all these values. An average migration directness value close to 0 is obtained if a cell moves totally random around one spot and a value of 1 is obtained if a cell moves away from its origin in a straight line. Thus, each cell has a direction of migration independent of the other cells.

Fig. 6 shows the motility of cells in relation to cell cycle time (cells that divide, upper panels) and observation time (cells that do not divide, lower panels). The motility was similar for L929 cells that divided either cultivated in normoxia (Fig. 6A, upper panel) or hypoxia (Fig. 6B, upper panel). The slightly positive slope of the lines indicates that motility increased with cell cycle time, which is expected as longer cell cycle times results in a longer time for cell movement. This was also true for cells with incomplete cell cycles, as seen in the lower panel of Figs. 6A and 6B. The same results were found for JIMT-1 cells cultivated in normoxia (Fig. 6C) or hypoxia (Fig. 6D). When treating
JIMT-1 cells cultivated in normoxia or hypoxia with 0.5 μM salinomycin (Fig. 6E and Fig. 6F, respectively), the motility of the cells, regardless of normoxic (Fig. 6E) or hypoxic (Fig. 6F) condition, increased significantly compared to control (p-value, $1.2 \times 10^{-9}$ and $2.3 \times 10^{-10}$, respectively). The linear relationship with the cell cycle time is still evident.

The mean average migration directness of cells with completed cell cycles was similar for L929 cells cultured in normoxia (Fig. 7A) or hypoxia (Fig. 7B). The mean average migration directness of cells with completed cell cycles was similar for control and salinomycin-treated JIMT-1 cells cultured in normoxia (Fig. 7C, top panel and Fig. 7D, top panel, respectively) or hypoxia (Fig. 7D, top panel and Fig. 7F, top panel, respectively). The distribution of average migration directness was broad (large confidence interval) and had no correlation with cell cycle time.

When JIMT-1 cells cultivated in normoxia or hypoxia were treated with salinomycin (Fig. 7E, bottom panel and Figure 4. Time to first and last division of each cell tree. Each triangle represents one division of the first (A-D) or last (E-H) generation of a cell tree. (A) L929 cells in normoxia and hypoxia. (B) JIMT-1 cells in normoxia and hypoxia. (C) JIMT-1 cells in normoxia cultured in the absence or presence of 0.5 μM salinomycin. (D) JIMT-1 cells cultured in hypoxia in the absence or presence of 0.5 μM salinomycin. (E) L929 cells in normoxia and hypoxia. (F) JIMT-1 cells in normoxia and hypoxia. (G) JIMT-1 cells in normoxia cultured in the absence or presence of 0.5 μM salinomycin. (H) JIMT-1 cells cultured in hypoxia in the absence or presence of 0.5 μM salinomycin. **P < 0.05, ***P < 0.005, ****P < 0.0005. The data are compiled from three experiments.

Table 2. Population doubling times and cell cycle times for L929 cells and JIMT-1 cells, cultivated in normoxia or hypoxia and treated with 0.5 μM salinomycin (JIMT-1 only).

|                    | Population doubling time (h) in time interval after seeding | Mean cell cycle time (h) in time interval after seeding | Percent of cells that divide during the 72–96 h time span. |
|--------------------|-------------------------------------------------------------|-------------------------------------------------------|----------------------------------------------------------|
|                    | 24–48 48–72 72–96                                         | 24–48 48–72 72–96                                      | Of all cells (%)                                         |
| L929 normoxia      | 15.5 26 36                                                | 14.2 15.1 17.1                                         | 23                                                       |
| L929 hypoxia       | 22 58 —                                                  | 11.7 19.4 28.6                                         | 3                                                        |
| JIMT-1 normoxia    | 51.5 19.5 27.5                                           | 17.1 20.8 22                                            | 36                                                       |
| JIMT-1 normoxia 0.5 μM salinomycin | 35 60 —                                                  | 19.8 26.3 37.8                                         | 5                                                        |
| JIMT-1 hypoxia     | 27 30 62.5                                               | 15.8 22.1 26.6                                         | 11                                                       |
| JIMT-1 hypoxia 0.5 μM salinomycin | 30 — —                                                   | 18.1 29.8 41.3                                         | 5                                                        |

*The population doubling times are deduced from Fig. 1.

*Mean cell cycle times for completed cell cycles. The population doubling times are deduced from Fig. 3A-F. Time 0 in tracking in Fig. 3 is equivalent to 24 h after seeding in Fig. 1. The SD is not shown in this table for simplicity. Please see supplement Table 1 for SD data.

*From cells tracked in HoloMonitor M3 and M4. Values from Fig. 3A-F.

*No population doubling time could be found.
Fig. 7F, bottom panel, respectively), the pattern of distribution of cells, with non-completed cell cycles, regarding average migration directness versus end of tracking changed compared to the pattern of cells not treated with salinomycin (Fig. 7C, bottom panel and Fig. 7D, bottom panel, respectively). There was a shift in the distribution of more

Figure 5. Motility and migration as defined in HStudio™. Motility (blue line) is the accumulated movement of the cell over time. Migration (red line) is the shortest distance from the starting point of the cell at time 0 and the current position for each time point, regardless of the movement in between the two time points.

Figure 6. The dependence of cell motility on cell cycle time and tracking time. Motility is defined as the total distance a cell has moved during the observation time. The upper part of each subfigure shows the motility versus cell cycle time. The lower part of each subfigure shows motility in relation to tracking time for cells with non-completed cell cycles. The symbols are described in Table 1 and Fig. 2. The mean motility is calculated for cells with completed cell cycles ± confidence interval at 95% confidence level. n is number of cells. Linear regression lines with their respective slopes are shown. Black regression lines represents the collected regression of orange, red, and purple X:s. (A) L929 cells cultured in normoxia. (B) L929 cells cultured in hypoxia. (C) JIMT-1 cells cultured in normoxia. (D) JIMT-1 cells cultured in hypoxia. (E) JIMT-1 cells treated with 0.5 μM salinomycin cultivated in normoxia. (F) JIMT-1 cells treated with 0.5 μM salinomycin cultivated in hypoxia. The data are compiled from three experiments.
cells towards a lower average migration directness compared to non-treated control in normoxia or hypoxia (Fig. 7C, bottom panel and Fig. 7D, bottom panel, respectively). We have previously shown that salinomycin treatment induces MET in JIMT-1 cells. Thus, we hypothesized that this shift maybe reflects a change from mesenchymal to epithelial phenotype. To test our hypothesis, we investigated the more epithelial-like breast cancer cell line MCF-7. In MCF-7 cells, the motility pattern was similar to that of salinomycin-treated JIMT-1 cells i.e. the cells showed a high degree of motility (Fig. 8A). However, the mean average migration directness was low, indicating that the increased motility did not result in distant migration (Fig. 8B).

In Fig. 9, the average migration directness versus observation time (i.e. cell cycle time or tracking time depending on fate) for all tracked cells are shown. When the mesenchymal JIMT-1 breast cancer cells cultured in normoxia or hypoxia were treated with salinomycin, the distribution of cells shifted to a distribution more resembling that of the epithelial MCF-7 breast cancer cells in normoxia i.e. an increased number of cells with a low average migration directness and long observation time.

**Discussion**

It has been increasingly acknowledged that the outcome of cancer therapy is determined by the response of drug treatment on the molecular level in individual cells. Thus, methods of longitudinal tracking are a powerful approach to understand the biology of individual cells and to observe cell-to-cell variability within a population and this contributes to knowledge about population response dynamics. In the present study, we used...
digital holographic microscopy achieving 72-h data from non-
labelled cells. Time-lapse phase contrast and immunofluores-
cence microscopy are most commonly used for longitudinal
cell tracking although in general for shorter time periods than
in our study.\textsuperscript{13,24-26} Another method used to track longitudinal
changes in mass and area of cell clusters under different growth
conditions is spatial light interference microscopy.\textsuperscript{27}

The population doubling time, obtained from growth
curves, is derived from both cycling and non-cycling cells. Our
data clearly show that there are sub-populations of rapidly
dividing cells hiding in population-based data such as the pop-
ulation doubling time. Thus, by only drawing conclusions
based on population data, important biological processes on
individual cell levels may be neglected. For instance, if only
observing the growth curve for L929 cells growing in hypoxia,
we would conclude that all cells have stopped dividing at 72 h
after seeding, while the time-lapse data show that there still are
cells dividing. Similar results have been reported for HeLa cells
using a spinning disc confocal microscopy system.\textsuperscript{29} Thus, data
on individual cell features are hiding within the data of the pop-
ulation, which is also apparent for JIMT-1 cells.

The L929 cells showed higher sensitivity towards the
hypoxic condition than the JIMT-1 cells. Differences in cell
proliferation in normoxia compared to hypoxia is also
apparent for other cells lines.\textsuperscript{29,30} Altogether these data
reflect a phenotypic and genotypic diversity in cell popula-
tions resulting in heterogeneity of cell responses to a com-
mon stimulus. Presently it is not possible to use digital
holographic imaging to accurately distinguish how differen-
cees in lengths of the individual cell cycle phases contribute
to heterogeneity in cell cycle times.

The growth inhibiting effect of salinomycin treatment has
been shown in numerous studies.\textsuperscript{4,7,31-33} However, this is the
first study of how salinomycin affects individual cells cultured
in normoxia or hypoxia during a 72 h time period. By monitor-
ing the times to first and last division in individual cell trees it
is possible to get an indication of sensitivity to different condi-
tions. In control, the length of time to the first division depends
on the variation in the cell cycle time of individual cells of a
population and the asynchrony of the population including if
there were cells in G\textsubscript{0} phase of the cell cycle when the tracking
was initiated. When cells are treated, the time to first division

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{Motility and average migration directness in human epithelial MCF-7 cells. The symbols are described in Table 1 and Fig. 2. Lines show linear regression with slope indicated. Black regression lines represents the collected regression of orange, red, and purple X:s. (A) Motility versus cell cycle time for MCF-7 cells in normoxia. (B) Avg. migration directness versus cell cycle time for MCF-7 cells in normoxia. The data are compiled from three experiments.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9}
\caption{The average migration directness versus observation time is similar in the epithelial MCF-7 breast cancer cell line (A) and in salinomycin-treated JIMT-1 cells cultured in normoxia (A) and hypoxia (B).}
\end{figure}
will also be affected by the molecular mechanism of the compound e.g. if there are cell cycle specific effects or non-specific effects. Our data show that treatment with 0.5 μM salinomycin had limited effect on ongoing cell cycle progression when the treatment started and that the daughter cells in the next generation were blocked in their cell cycle progression. Salinomycin is well-documented regarding CSC specificity in population-based studies.4,7,24,35 Although the present study investigates the response of individual cells to salinomycin treatment, we cannot yet distinguish cells with different phenotypes using digital holographic imaging. We can only speculate that the early responder cells (i.e. a halted daughter cell after division) and cells that never divide, maybe are CSCs and are presently investigating this notion combining digital holography with fluorescence microscopy. Our previous work has shown that treatment of JIMT-1 cells with 0.5 μM salinomycin reduces the CSC population around 50%.7 The phenotype of JIMT-1 cells still proliferating after 72 h of treatment with 0.5 μM salinomycin remains to be elucidated as well as their sensitivity to other chemotherapeutic drugs. A synergistic effect between salinomycin and conventional chemotherapeutic drugs that target cycling cells has been demonstrated26,37 as well as the development of resistance to salinomycin.38

When JIMT-1 cells were treated with salinomycin, motility increased significantly. At a first glance this is contrary to a number of observations showing that salinomycin treatment decreases cell movement as evaluated in wound healing and Boyden chamber assays.5,7,39-42 However, those assays evaluate migration from a start point to an end point, while motility is a measure of how much cells are moving, independent of how far they migrate from the starting point. Thus, a cell can have very high motility but migrate a very short distance, i.e. move around in a small spot. Taking into account the distance the cells move by using the parameter average migration directness, it is obvious that salinomycin treatment, despite inducing increased motility, resulted in decreased migration as has been reported previously. We have shown that salinomycin treatment-induced decrease in cell migration is accompanied by increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal marker vimentin in JIMT-1 cells, indicating epithelial transition to MET to epithelial transition.7 Thus, we decided to investigate motility and migration in the MCF-7 breast cancer cell line, which has more epithelial features than the JIMT-1 cell line. MCF-7 cells grow in tighter colonies than JIMT-1 cells and MCF-7 cells have higher expression of the epithelial markers E-cadherin and β-catenin at the cell surface.43 Our data show that, similar to salinomycin-treated JIMT-1 cells, the MCF-7 cells had a high motility, but low average migration directness. Thus, based on our data we propose that MET and EMT can be investigated by longitudinal cell tracking of motility and migration. However, this notion must be more thoroughly investigated using normal mesenchymal and epithelial cells.

In conclusion, our data show that there are individual cell responses, regarding cell cycle length and progression and cell movement behaviour, upon treatment with salinomycin of cells cultured under normoxic or hypoxic conditions that were not to be found in population based data. Thus, methods for longitudinal investigation of cell fates during different treatment conditions, is an important tool to understand the heterogeneity in responses and most importantly to guide cutting-edge drug development.

Materials and methods

Cell culturing

The experiments were conducted using three cell lines: the human breast cancer cell line JIMT-1 (ACC-589) was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), the human breast cancer cell line MCF-7 (ATCC® CCL-1™) and the mouse fibroblast cell line L929 (ATCC® CCL-1™) were purchased from American Type Culture Collection (Manassas, VA, USA).

JIMT-1 cells were routinely cultured in DMEM:Ham’s F-12 (1:1) medium containing 10% fetal calf serum (VWR, Lund, Sweden), 1 mM non-essential amino acids (VWR), 100 U/ml penicillin (VWR), 100 μg/ml streptomycin (VWR), 2 mM L-glutamine (VWR) and 10 μg/ml insulin (Sigma-Aldrich, Stockholm, Sweden). MCF-7 cells were routinely cultured in RPMI 1640 medium (VWR), supplemented as the JIMT-1 cell medium. L929 cells were routinely cultured in RPMI 1640 medium containing 5% donor horse serum (Sigma-Aldrich), 1 mM non-essential amino acids, 1 mM Na-pyruvate (VWR), 50 U/ml penicillin, 50 mg/ml streptomycin, 10 μg/ml insulin and 1 mg/ml hydrocortisone. All cell lines were routinely passaged twice a week. The cells were kept in an incubator with 5% CO₂ in air at 37 °C.

For time-lapse experiments, cells were seeded in Petri dishes (3.5 cm diameter, Sarstedt, Nümbrecht, Germany) at a density of 7300 cells/cm² in 3 ml of regular growth medium, or in 25 cm² cell culture flasks (Nalgae Nunc International, Penfield, New York, USA) at a density of 15000 cells/cm² in 5 ml of regular growth medium. For growth curve experiments, the cells were seeded in 3.5 cm Petri dishes (Sarstedt) at a density of 15000 cells/cm² in regular growth medium. The lower cell density for the time-lapse experiments have previously been evaluated as a proper number to prevent a too confluent image over time, since this would affect the ability to track the cells. The different cell densities did not affect the result (not shown).

Treatment in normoxia or hypoxia

After seeding, cells were incubated in normoxia for 24 h to allow attachment. For experiments in hypoxia, the Petri dishes were then moved to a hypoxia chamber (DonWhitley Scientific, West Yorkshire, UK) with an atmosphere containing 1% O₂, 5% CO₂ and 60% humidity. The medium was changed to a medium equilibrated with normal DMSO concentration of 0.5 μM salinomycin giving a final DMSO concentration of 0.2%. Control cells were exposed to 0.2% DMSO.
**Growth curve experiments**

For growth curve experiments, cells were harvested by trypsinization (0.05% trypsin and 0.5 mM EDTA) and kept on ice before they were manually counted in a hemocytometer. The cell number was determined 24, 48, 72, and 96 h after seeding. All experiments were repeated at least three times.

**Digital holography time-lapse imaging and tracking**

HoloMonitor M3 and M4 (Phase Holographic Imaging AB, Lund, Sweden) are quantitative phase imaging systems based on digital holographic microscopy.\(^ {19,44,45}\) The images acquired from the systems are the digital reconstruction of the cells imprinted by the laser on a CCD-camera.\(^ {46}\) In the HoloMonitor system, a low power laser (635 nm wavelength, 0.2 mW/cm\(^2\)) is split into a reference and an object beam. The object beam is directed through the sample with cells, creating a phase shift in the laser beam, while the reference beam is kept undisturbed. Thereafter, the two beams are merged together creating an interference pattern, the hologram, that is projected on a CCD camera. Based on the hologram, a cell image is computationally reconstructed and can be used for analysis of e.g. cell morphology, cell movement and cell division.

Cells used for time-lapse imaging were placed on the HoloMonitor M3 or M4, immediately after the addition of the compound or the diluent (control) (in both normoxic and hypoxic conditions). Images were acquired with the software Hstudio\(^ {\text{TM}}\) (Phase Holographic Imaging AB) at the same position in the cell culture vessel every 5th minute for 72 h. One representative time-lapse movie per treatment is found in supplementary figures and statistics of e.g. cell morphology, cell movement and cell division.

Cells used for time-lapse imaging were placed on the HoloMonitor M3 or M4, immediately after the addition of the compound or the diluent (control) (in both normoxic and hypoxic conditions). Images were acquired with the software Hstudio\(^ {\text{TM}}\) (Phase Holographic Imaging AB) at the same position in the cell culture vessel every 5th minute for 72 h. One representative time-lapse movie per treatment is found in supplementary information (7 movies altogether). After image acquisition, the time-lapses were analysed by individual tracking of the cells using Hstudio\(^ {\text{TM}}\). The tracking is semi-automated. The algorithm attempts to find each tracked cell in the next frame by selecting the closest cell based on the centroid position. The user has to go through each individual captured image of the time-lapse and correct potential errors in the tracking. The data was used to calculate cell cycle time, motility, and average migration directness. Time of tracking equals time of treatment in all figures.

**Statistics**

The computer language R was used for creating figures and statistical analysis (R Core Team, 2015). Unpaired Students’ t-test was used to detect differences between treated and control. Regression lines are plotted in Figs. 3, 6, and 8. The slope of the lines is calculated and presented in each plot.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| CSC          | Cancer stem cell |
| MET          | Mesenchymal to epithelial transition |
| EMT          | Epithelial to mesenchymal transition |
| DHM          | Digital holographic microscopy |

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Authors’ contributions**

SK did the growth curve and analysed the time-lapse movies. SK and SO prepared the HoloMonitor for imaging. DS synthesized the salinomycin. SO, KA, BJ, and SK conceived the study, its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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