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GEFITINIB ENHANCED CANCER DRUG UPTAKE IN THE SAME SINGLE NON-SMALL CELL LUNG CANCER CELLS OBSERVED IN REAL-TIME IN THE MICROFLUIDIC BIOCHIP

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ABSTRACT

For high selectivity in cancer therapy, molecular-targeted drugs such as gefitinib are used. But there is drug resistance to its use on non-small cell lung cancer (NSCLC). In this case, chemotherapeutic drugs such as paclitaxel (PTX) will be used. However, multidrug resistance (MDR) occurs when the cancer cells resist chemotherapeutic drugs by pumping them out of the cells. MDR inhibitors such as cyclosporine A (CsA) can block MDR protein action and thus enhance the drug uptake in the cancer cells. This enhancement is clearly observed in real-time by conducting the same single cell analysis (*SASCA*) using a microfluidic biochip. The NSCLC cells such as NCI-H1650 were used for the study. By the *SASCA* method, the NCI-H1650 cell was found to have low initial PTX accumulation, and the treatment of the same lung cancer cell with PTX in the presence of CsA notably enhanced drug accumulation, indicating CsA is a MDR inhibitor. Moreover, gefitinib is found to be a MDR inhibitor and it enhances the drug accumulation in NCI-H1650 cells too. Therefore, we hypothesize when both paclitaxel and gefitinib are used in combination, NSCLC cells can be killed, whether they are gefitinib-resistant or multidrug resistant or not.

Keywords: Targeted drugs, lung cancer cells, multidrug resistance, fluorescent measurement, same single cell control.

INTRODUCTION

Resistance to a broad range of anticancer drugs, termed as multidrug resistance (MDR), is most often associated with the drug-pumping action of transporter proteins present on cancer cell membranes, leading to low cellular drug accumulation. One of the most important MDR mechanisms is an increased efflux rate of anticancer drugs from cancer cells by members of the ATP-binding cassette (ABC) transporters superfamily (Chen *et al*., 2016; Zhang *et al*., 2016). P-glycoprotein (P-gp) is the most common MDR transporter protein that plays a major role in cellular MDR via the efflux of different classes of chemotherapeutic drugs, and ultimately reduces the effectiveness of chemotherapy (Sharom, 2008; Sharom, 2011; Massey *et al*., 2014; Dlugosz and Janecka, 2016; Li *et al*., 2016). In this regard, blocking the MDR protein activity using MDR inhibitors is an important approach to overcome drug efflux. The MDR inhibitors can bind to the MDR proteins and stop them from functioning, ultimately resulting in the reversal of drug efflux (Wu *et al*., 2008; Klukovits and Krajcsi, 2015; Beretta *et al*., 2017).

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The drug uptake in MDR cells is poor; this has led us to study real-time drug accumulation in these cells. As we have observed in the same single cells within a microfluidic biochip, MDR inhibitors, which can prevent the drug transporters from pumping, have resulted in the immediate increase in the amount of anticancer drugs accumulated in the cells (Khamenehfar *et al*., 2014).

Lung cancer remains the leading cause of cancer death, and non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases (Chang 2011; Wangari-Talbot *et al.*, 2013). Gefitinib is designed as an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) for molecularly targeting NSCLC that has EGFR mutations (Paez *et al*., 2004).

In our study, we used two NSCLC cell lines, which are NCI-H1650 and NCI-HCC827. The former gefitinib-resistant cell line is reported to be much more resistant to gefitinib than the latter gefitinib-sensitive line, though both cell lines have the same kind of EGFR activating mutation (Choi *et al.*, 2010). Although it is still controversial whether MDR is present in NCI-H1650 cells, this mechanism might be involved in low

accumulation of gefitinib and its low TKI activity on the cell line. On the other hand, gefitinib has been reported to inhibit the function of P-gp on lung cancer cells, such as PC-6/PTX (Kitazakia *et al*., 2005; Nakamura *et al*., 2005). Therefore, we are interested to investigate the action of gefitinib as a P-gp inhibitor to enhance drug uptake in NCI-H1650 cells.

Here, a microfluidic biochip was used to measure the effect of gefitinib on enhancing drug accumulation in the same single NSCLC cells (NCI-H1650 and NCI-HCC827). We employed a method called same single cell analysis (*SASCA*) to measure drug accumulation in the microfluidically retained NSCLC cell (Khamenehfar *et al*., 2014). *SASCA* is a same single cell method that is a more powerful technique in identifying changes than conventional different single cell methods such as flow cytometry. This latter method requires 100,000 cells or more in order to definitively identify the change between the averaged drug accumulations in two distinct groups, i.e. (inhibitor + drug)-test group versus drug-only control. On the other hand, *SASCA* can identify the change between the drug accumulations on the same single cell treated first with drug only and then with drug + inhibitor, and can quickly report the change, as a fold-increase, in drug accumulation due to the action of the inhibitor (Khamenehfar *et al*., 2015; Khamenehfar *et al*., 2016). Furthermore, *SASCA* provides cellular morphological information which is collected simultaneously during the drug accumulation measurement. This information is useful to indicate the health and viability of the measured single cell. This method, conducted on the microfluidic chip, has been useful in identifying new MDR inhibitors from herbal ingredients (Chen *et al*., 2014).

MATERIALS AND METHODS

Experimental

Microchip design

The layout of the microfluidic chip was designed using AutoCAD (AutoDesk), and the chip design was sent to a printer (Coles Lithoprep) which produced the photomask on a plastic film. The microfluidic chip was made of polydimethylsiloxane (PDMS) that was sealed to a 0.17-mm glass cover slip. As shown in Figure 1, the microfluidic PDMS chip (15 mm x 15 mm) consists of five reservoirs and one chamber containing the cell retention structure. The left and right reservoirs (1, 5) serve as the inlet and waste reservoirs, respectively; whereas, the top reservoirs (2, 3, 4) are used for drug delivery. The cells were selected by hydrodynamic liquid flow. The channel depth was 40 μm, while the reservoirs were 0.6 mm deep and 2 mm in diameter.

This chip was different from the ones described previously (Li *et al*., 2009; Khamenehfar *et al*., 2014) in that it was made of polymeric plastics. The cell retention structure located inside the chamber was used to select and retain a single cell. Based on this active cell trapping strategy, we were able to select and retain a cell in a good condition and flush away any undesired cells or debris.

Cell Culture

NCI-H1650 and NCI-HCC827 lung cancer cells were obtained from ATCC. They were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2 mM glutamine, as previously reported (Fan *et al*., 2014). For subculture, the cells were detached using trypsin-EDTA and re-seeded. All cells were cultivated at 37 °C in a 5% CO_2 incubator.

Reagents

Gefitinib, Oregon Green-labeled paclitaxel (OG-PTX or Flutax-2) and cyclosporine A (CsA) were obtained from Sigma-Aldrich (St Louis, MO). A stock solution of gefitinib (4.5 mM) was prepared in dimethylsulfoxide (DMSO) and it was diluted to the final concentration of 1-10 μM (Cheng *et al*., 2011). OG-PTX was dissolved in DMSO to make a stock solution of 1 mM and it was diluted to a final concentration of 3 μM. CsA was dissolved in DMSO (Sigma-Aldrich) to make stock solutions of 500 μM and it was diluted to the final concentration of 5 μM.

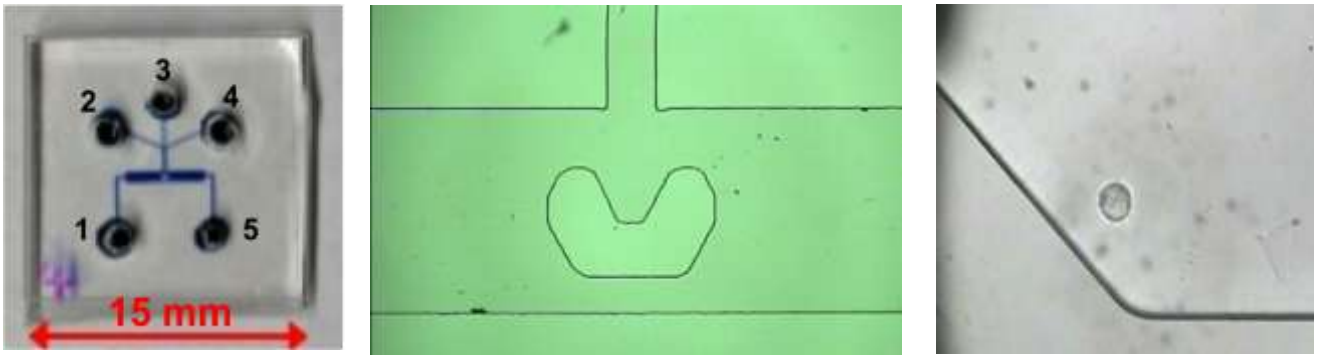
On-Chip Drug Accumulation Study

A single cancer cell was selected from within a microfluidic biochip for *SASCA* measurements, as previously described (Li *et al*., 2009). Briefly, after several cells were introduced from reservoir 1 (inlet reservoir), solutions from all other reservoirs were removed; therefore, the cells flowed from left to right inside the chamber. The desired cell moved further and passed the entrance of the cell retention structure. After adding solution from reservoir 5 (outlet reservoir), the cell moved back to the entrance of cell retention. Then, the cell was pushed into the cell retention structure by inducing flow via reservoirs 2, 3 and 4, which were connected to central reagent channel. The trapped cell was settled for ~ 15 min before the fluorescence measurement started. The biochip was mounted on a microscope stage for fluorescent measurement and live cell imaging simultaneously. For the *SASCA* experiment, the first step was the accumulation of the anticancer drug (i.e. OG-PTX) in the single NSCLC cell measured in the absence of the gefitinib. In the next step, in the same cell, drug accumulation was measured in the presence of an MDR inhibitor compound. Adding MDR inhibitors increased drug accumulation, and then the single cell fluorescence intensity was enhanced. OG-PTX was used for drug accumulation measurement since it was a substrate of the P-gp, and the labeled drug also has fluorescence (λ_{ex}= 488 nm; λ_{em}= 524 nm). The excitation light was provided by

the xenon arc lamp, which was not critical to provide an impact on the cell membrane permeability.

During data collection, the chip was moved back and forth across the detection aperture window: briefly, when the cell was inside the detection window, the cellular fluorescence was measured; whereas the background signal was measured when the cell was outside the detection window. Subtraction of the background from the cell signal intensity gave a corrected signal representing the drug concentration inside the cell.

The experiments were first conducted on the NCI-H1650 cells, followed by the NCI-HCC827 cells. In the experiments, the single cell was first treated with the anticancer drug (3 μM of OG-PTX) in the absence of MDR inhibitors (~1000 s) for drug accumulation (control experiment). In the next step, in the same single cell, the enhanced drug accumulation was measured in the presence of a P-gp inhibitor at different concentrations (~1000 s for each step). Gefitinib was used at different concentrations for its ability to enhance drug accumulation in MDR cells. Cyclosporine A, a well-known P-gp inhibitor (Qadir *et al*., 2005), was used as the positive control.



(a) *(b)* *(c)*

Fig. 1. The microfluidic biochip. (a) Image of the microchip with channels filled with a blue food dye for easy visualization. PDMS chip showing reservoirs 2, 3, 4 used for drug delivery, reservoirs 1 as the cell inlet and reservoir 5 as the waste, (b) region of the central chip chamber showing the cell retention structure, (c) The close-up of the chamber with cell retention structure. As shown, a single NCI-H1650 cell retained within the retention structure

RESULTS AND DISCUSSION

A single NCI-H1650 cell was first captured for measurement (Fig. 1c). For fluorescence measurement, the initial signal obtained from 3 μM of OG-PTX was low but sufficient. Figure 2 shows drug accumulation in this cell to be evaluated in the absence and presence of different concentrations of gefitinib (1, 5, 10 μM). This is followed by treating the same cell with CsA (5 μM), which is a positive control of a MDR inhibitor. The effectiveness of the MDR inhibitor is indicated by the fold-increase in fluorescence, which is defined as the ratio of the fluorescence signal of the inhibitor-blocked cell to that of the unblocked cell (the control). As shown in Figure 2a, the addition of different concentrations of gefitinib (1, 5, 10 μM) notably increased the drug accumulation in the cell and caused the single cell fluorescence to increase by 1.7, 3.2 and 3.5 fold, respectively, in comparison to the unblocked cell treated with OG-PTX only as the control.

As shown in Figure 3, a similar experiment was performed on the other NCI-H1650 single cell by treating it with 10 μM gefitinib directly and for a longer time. Figure 3a illustrated the data trace started with the measurement of cell background (at 50 s), followed by the introduction of OG-PTX (at 100 s). The background level has increased due to the fluorescence of OG-PTX; the subsequent increase in the total fluorescence signal was due to the accumulation of the drug in the cell. The cell signal, which can be obtained by subtracting the background from the total signal (Peng *et al*., 2005), is believed to relate to the drug concentration in the cell. As soon as gefitinib was added to the cell (at 1000 s), an increase in cell fluorescence, which indicated an enhanced drug accumulation in the cell, was observed immediately (at 1080 s), with a final fold-increase stood at 3.2. This number is encouraging, as compared with the value of 1.9 calculated by dividing the fluorescent indices of rhodamine-123 accumulation in PC-6/PTX cells with and without 10 μM gefitinib, as reported in a previous flow cytometric study (Kitazakia *et al*., 2005). The fold-increase value for CsA, as calculated from values obtained in the same report, is 4.0 which is consistent with the fold-increase of 4.0 obtained in our study. Figure 2b shows the images of the same cell taken at different stages of the *SASCA* experiment. This cellular morphological information, which is collected simultaneously during the drug accumulation measurement, is useful to indicate the membrane integrity and cell viability of the measured single cell.

Although NCI-H1650 is known to be resistant to the TKI activity of gefitinib, its effect on enhancing the

accumulation of OG-PTX in the cell is demonstrated, which corroborates with a previous report (Kitazakia *et al*., 2005). This additional effect of gefitinib to enhance drug accumulation may provide an alternative mechanism to cause death of gefitinib-resistant NSCLC cells by the use of the anti-cancer drug PTX.

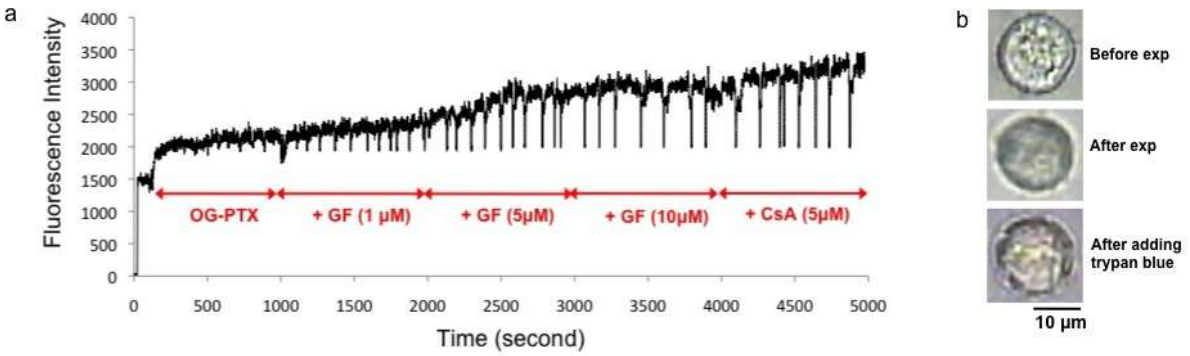


Fig. 2. Drug accumulation in a single NCI-H1650 cell in the presence of gefitinib, followed by CsA.
(a) Fluorescent intensity measured in real time. The fold-increase after adding 1, 5, 10 μM of gefitinib was 1.7, 3.2, and 3.5, respectively; that for 5 μM of CsA was 4.0. (b) The cell images were depicted before and after experiment (exp), followed by adding trypan blue to check for cell death. Scale bar: 10 μm.
OG-PTX: Oregon Green-labeled paclitaxel (3 μM); GF: gefitinib; CsA: cyclosporine A.

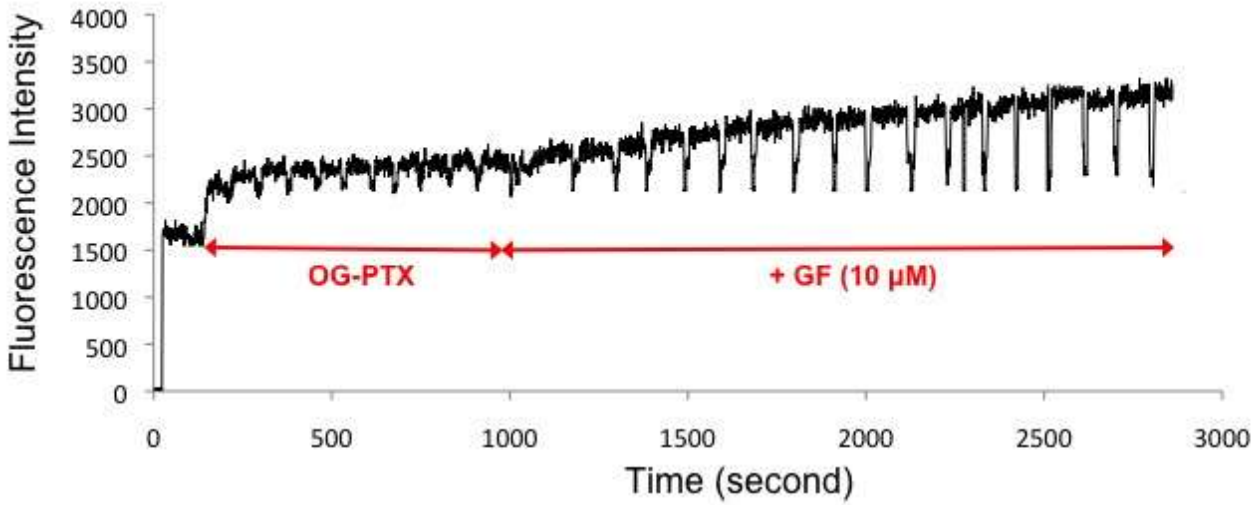


Fig. 3. Drug accumulation in a single NCI-H1650 cell in the presence of gefitinib. OG-PTX: Oregon Green-labeled paclitaxel (3 μM).The fold increase after adding 10 μM of gefitinib (GF) was 3.2.

Conducting the same *SASCA* procedure on the single gefitinib-sensitive NCI-HCC827 cell did not result in any fluorescence enhancement due to gefitinib (see Fig. 4). Even the use of CsA did not result in any fold-increase. This negative result may be attributed to the presence of non-Pgp drug transporters, which were not blocked by CsA. In any event, for this gefitinib-sensitive cell, since gefitinib can inhibit the tyrosine kinase activity of the cell, the use of the targeted drug should be sufficient to kill it.

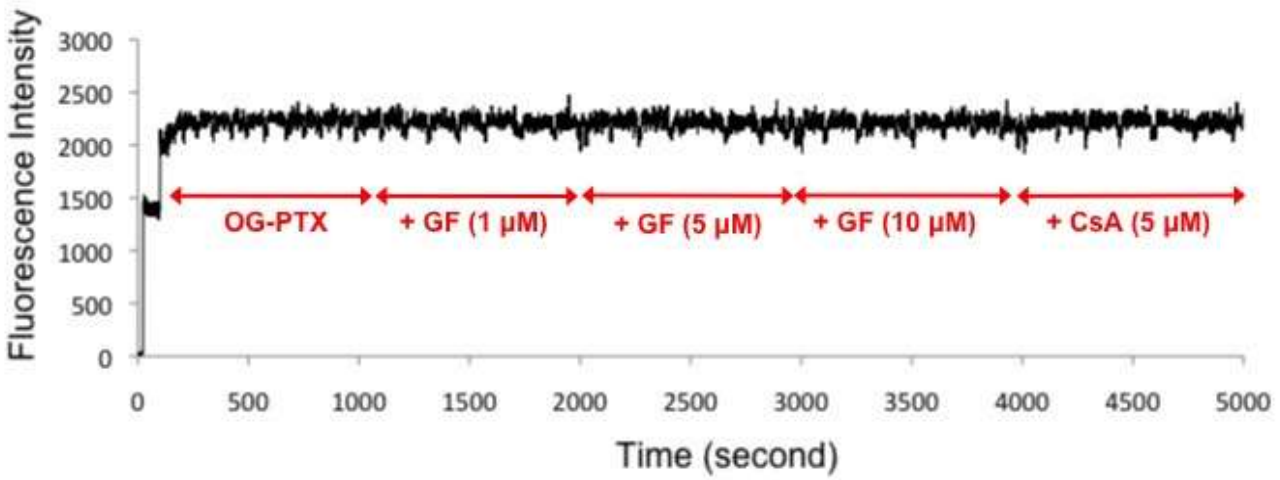


Fig. 4. Drug accumulation in a single NCI-HCC827 cell in the presence of gefitinib, followed by CsA. OG-PTX: Oregon Green-labeled paclitaxel (3 μM). No fold-increase observed after adding different concentrations of gefitinib, followed by CsA.

CONCLUSIONS

The microfluidic *SASCA* method has provided time-dependent drug transport data in single non-small cell lung cancer (NSCLC) cells as well as the cell morphological information. This same single cell method provides an immediate observation of the enhancement of cellular drug accumulation due to the action of the P-gp inhibitor. Furthermore, only a small amount of cells and reagents are needed to confirm the findings. The ability of gefitinib in enhancing drug accumulation in the NCI-H1650 cell suggests an alternative mechanism to kill this type of gefitinib-resistant NSCLC cell. It has been reported that PTX and gefitinib are used in combination for lung cancer treatment (Cheng *et al*., 2011), and our study suggests that the gefitinib-resistant and MDR non-small cell lung cancer cells, such as NCI-H1650, has an enhanced accumulation of PTX due to the gefitinib action.

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