An integrated microfluidic cell array for apoptosis and proliferation analysis induction of breast cancer cells

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In vitro sensitivity testing of tumor cells could rationalize and improve the choice of chemotherapy and hormone therapy. In this report, a microfluidic device made from poly(dimethylsiloxane) and glass was developed for an assay of drug induced cytotoxicity. We evaluated the apoptotic and proliferation-inhibitory effects of anticancer drugs mitomycin C (MMC) and tamoxifen (TAM) using MCF-7 breast cancer cells. MMC and TAM both induced apoptosis and inhibited proliferation of MCF-7 cells in a concentration-dependent manner. MMC caused the expression of antiapoptotic protein Bcl-2 a dose-dependent reduction in MCF-7 cells. The expression of Bcl-2 did not change significantly in MCF-7 cells treated by TAM. The results in the microfluidic device were correlated well with the data obtained from the parallel experiments carried out in the conventional culture plates. The developed microfluidic device could be a potential useful tool for high content screening and high throughput screening research. © 2010 American Institute of Physics. [doi:[10.1063/1.3497376](https://doi.org/10.1063/1.3497376)]

I. INTRODUCTION

The various protocols of chemotherapy and hormone therapy for breast cancer have different and limited rates of success.¹⁻³ One potential approach to improve the therapeutic efficacy is to use *in vitro* approaches to evaluate the sensitivity of agents using patients' tumor cells. The balance between programmed cell death (apoptosis) and cell proliferation determines the tumor growth rate, and alteration between these two factors may be the key element for the uncontrolled expansion of malignant tumors. Current methods to evaluate the sensitivity of agents are generally expensive and labor-intensive, utilizing multiwell plates that are operated using cumbersome manual or expensive robotics-based operations. Therefore, it is of interest to develop a technology that can perform such experiments in a cheaper, easier, and higher throughput manner.

Microfluidic chips have been applied for cell sorting, cell patterning and immobilization, cell culture and development, patch clamp, electroporation, and cell-cell signal detection.⁴ The advantages of microfluidic chips including ease of integration and the potential for parallel processing make it to be an attractive platform for drug metabolism and cell cytotoxicity analysis.⁵ Since a variety of conditions can be screened simultaneously, it allows efficient exploration of a wide parameter space for molecular stimulation of cells. Wang *et al.*⁶ developed a high-density microfluidic array platform for high-throughput cell cytotoxicity screening. Wlodkowic *et al.*⁷ developed a microfluidic array for the real-time screening of anticancer drugs against arrays of single cells. Human HL60 cells were used to quantify on-chip anticancer drug induced apoptosis. The integration of drug metabolism bioreactors and microscale cell culture elements allows drug metabolite characterization and cytotoxicity assays simultaneously on a single microfluidic device.⁸

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Therefore, microfluidic chips can be a novel cost-effective technology for drug discovery.⁹ Since the majority of anticancer drugs presently used in a clinical setting have been described to both induce cell apoptosis and inhibit cell proliferation, however, until date, cell-based multiparameter screening, which integrated both cytotoxicity assessments and cell proliferation, has not been established yet in the microfluidic chips.

In the present study, we described microfluidic device-based multiparametric assessment of cellular responses in MCF-7 cells induced by anticancer drugs. The simple assay provided multiparametric measurements of phosphatidylserine (PS), plasma membrane permeability, nuclear morphology, and the expression of antiapoptotic protein Bcl-2 or Ki-67 (a marker of proliferation) during drug-treated MCF-7 cells. This work provided us with valuable basis to develop a microfluidic chip for high content and high throughput drug screening and to improve cancer treatment.

II. MATERIALS AND METHODS

A. Microfluidic device fabrication

The microfluidic device consists of the flow channels with $40\ \mu\text{m}$ (h) $\times$ $100\ \mu\text{m}$ (w) and eight circular chambers at each channel (diameter of $400\ \mu\text{m}$). Within the circular chambers in the flow layer are eight poly(dimethylsiloxane) (PDMS) U-shaped cell sieves. Each sieve is semicircular, $80\ \mu\text{m}$ (d), $20\ \mu\text{m}$ (w), and $40\ \mu\text{m}$ (h) (equivalent to the flow layer height), with two apertures ($8\ \mu\text{m}$). The structure was fabricated from PDMS (Sylgard 184, Dow Corning) using replicate molding and soft lithography. SU-8 50 (Microchem, USA) was patterned on a silicon wafer using high-resolution design transparencies to create the distinct positive-relief casting mold for the elastomer. The mold was subsequently exposed to vapor phase chlorotrimethylsilane (Aldrich) to facilitate the release of the elastomer during molding. Sylgard 184 liquid silicone elastomer (mixed in a ratio of 10 part A:1 part B) was poured on the mold ($\sim 5\ \text{mm}$ thick), and baked at $80\ ^\circ\text{C}$ for 30 min. After baking, the cured PDMS layer was peeled from the mold, fluid inlet and outlet ports were punched using a 23 gauge luer stub (BD Biosciences). The round inlet and outlet have a diameter of 0.75 mm. To remove residue generated during the punching process, the microchannels and connection ports were cleaned with isopropanol, dried with nitrogen, and the exposed microchannel surface on the molded device was irreversibly bonded to a glass slide using oxygen plasma (150 mTorr, 50 W, 20 s).

B. Cell culture

The MCF-7 cell line was purchased from ATCC (Manassas, VA, USA). The cells were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) with high glucose (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS), containing $100\ \text{U ml}^{-1}$ penicillin and $100\ \text{U ml}^{-1}$ streptomycin in $25\ \text{cm}^2$ flasks (Corning). The cells were maintained in a 5% CO_2 humidified incubator at $37\ ^\circ\text{C}$. Once the cells were confluent, they were trypsinized (0.25% in ethylenediamine tetraacetic acid, Hyclone) and passaged at a 1:4 subculture ratio. MCF-7 cells were cultured in flasks for 2–3 days prior to microfluidic experiments to ensure the cells used for experiments in exponential growth phase.

C. Cell seeding and culture in microfluidic device

The operation of the microfluidic device for drug-induced apoptosis and antiproliferation included cell seeding, cell culture, drug stimulation, fluorescence staining, and image analysis. Prior to cell culture, the microfluidic device was sequentially washed with dehydrated alcohol and water and then baked at $80\ ^\circ\text{C}$ overnight. On the next day, the flow channel glass surface was precoated with $20\ \text{mg ml}^{-1}$ gelatin (Sigma-Aldrich Co., St. Louis, MO, USA) for 1 h to promote cell attachment. Excess gelatin was removed by rinsing with $1\times$ phosphate buffered solution (PBS). A hemocytometer was used to assess the culture density of MCF-7 cells. After counting, the cells were spun down in a centrifuge (1000 rpm, 5 min) and reconstituted at $10^6\ \text{cells ml}^{-1}$ in DMEM supplemented with 10% FBS. To load the cells into the circular cell culture chambers, the cells ($\sim 10^6\ \text{cells ml}^{-1}$, $1\ \mu\text{l min}^{-1}$ volumetric flow rate, 3 min total load time) were injected into

the device through the inlets using a 24-channel roller pump (ISMATEC).¹⁰ The seeded microfluidic device was maintained at 37 °C/5% CO₂ overnight for proper cell attachment and spreading. Once the cells reached 70% confluency, the culture medium without and with mitomycin C (MMC) or tamoxifen (TAM) at 2, 10, and 50 μM was simultaneously perfused into the microfluidic device through the inlets, using a 24-channel roller pump (ISMATEC), culture medium was used as a drug-free control. The microfluidic device was then incubated in an incubator at 37 °C/5% CO₂. After 24 h, the drug exposure was stopped and specific fluorescent dyes or antibodies were selected for identifying apoptosis or proliferation markers. Control parallel experiment was performed by static exposure of TAM, under the same concentrations and exposure time. To maintain the static situation of media plus TAM, the outlets of the microfluidic device were shut down.

D. Analysis of apoptosis using fluorescent staining

The cells in microfluidic devices were treated with MMC or TAM as described previously. After 24 h drug exposure, the cells were washed with phosphate buffered solution containing calcium ion (DPBS), and then double labeled with fluorescein isothiocyanate (FITC)-labeled Annexin-V and propidium iodide (PI) using an apoptosis kit (Invitrogen, USA) in accordance with the manufacturer's instructions. In order to count the number of total cells in each chamber, all the cells were costained with 5 μg/ml Hoechst 33258 (Sigma-Aldrich Co., USA). Using the Annexin-V-PI double staining regime, three populations of cells were distinguishable: (1) nonapoptotic cells: Annexin-V negative and PI negative; (2) early apoptotic cells: Annexin-V positive and PI negative; and (3) necrotic cells or late apoptotic cells: Annexin-V positive and PI positive.

E. Immunofluorescent staining of Bcl-2 and Ki-67

After 24 h drug exposure as described above, the cells were washed with DPBS before being stained. The cells were then fixed with 4% m/v paraformaldehyde in PBS for 10 min followed by rinsing with DPBS, permeabilized with 0.1% v/v Triton X-100 in PBS for 15 min then washed with DPBS. 1% m/v bovine serum albumin in PBS was used before the detection of Bcl-2 and Ki-67 with immunofluorescent staining. After blocking, the medium with FITC-labeled anti-Bcl-2 (0.2 μg/μl; BD, USA) or FITC-labeled anti-Ki-67 monoclonal antibody (0.2 μg/μl; Santa Cruz Biotechnology, Inc., USA) at a dilution of 1:100 (v/v) was introduced into the culture chamber, incubated at 37 °C for 1 h. To enumerate the total cells in each chamber, the cells were costained with Hoechst 33258 (5 μg/ml Sigma-Aldrich Co., St. Louis, MO, USA). After the wash, the cells were imaged immediately. As comparison, MCF-7 cells were also seeded into four-well chamber slide (Nunc™ Lab-Tek®, USA) at 5×10^4 cells ml⁻¹ in 500 μl culture medium per well, and treated with same doses of MMC or TAM as applied in the microfluidic device.

F. Image acquisition and analysis

Specific fluorescent dyes or antibodies were used to identify apoptosis or proliferation markers in MCF-7 cells. The stained cells were imaged by an inverted fluorescence microscope (Olympus IX 71, Japan). The microfluidic device was mounted on the microscope equipped with a motorized X-Y stage. Objective lens (×20) and appropriate fluorescence filters, i.e., 488 nm excitation/530 nm emission for FITC, 525/595 nm for PI, and 356/465 nm for Hoechst 33258, were used for obtaining multicolor images, which were overlaid as required. The numbers of Annexin-V positive cells, PI positive cells, Hoechst positive cells, Ki-67 positive cells, and the average optical density of Bcl-2 in each chamber were measured using IMAGE-PRO software (Media Cybernetics, USA). The percentage of cell apoptosis and proliferation was calculated to evaluate the effects of MMC or TAM in MCF-7 cells. These measurements are recognized as necessarily semiquantitative; however, all measurements were made under the same conditions so that comparisons between samples could be made.

FIG. 1. Schematic of the 16×8 chamber microfluidic device. (a) Computer-aided design schematic of the 16×8 chamber microfluidic device. (b) Image of the microfluidic device with fluid interconnects. (c) Each chamber contains eight microsieves for cell trapping. Each microsieve is semicircular with an interior diameter of $80\ \mu\text{m}$, $40\ \mu\text{m}$ (h), and $20\ \mu\text{m}$ (w) with two apertures ($8\ \mu\text{m}$ width). (d) MCF-7 cells culture in the microfluidic device at a magnification of $\times 200$. Scale bar is $100\ \mu\text{m}$.

G. Cell cytotoxicity assay using MTT assay

Cell viability in 96-well plates was determined by the methyl thazolyl tetrazolium (MTT) assay. In brief, cells were seeded at 10^4 cells per well in 96-well plates. After drug exposure, $20\ \mu\text{l}$ of $5\ \text{mg/ml}$ MTT was added and plates were incubated at $37\ ^\circ\text{C}$ for 4 h. Wells were drained and formazan crystals were solubilized in $150\ \mu\text{l}$ dimethyl sulphoxide (DMSO). The optical densitometry was measured at a wavelength of $550\ \text{nm}$ (reference $630\ \text{nm}$). Percentage survival was determined as per the formula (absorbance of drug-treated cells/absorbance of control cells) $\times 100$ and compared with control, untreated cells regarded as 100%.

H. Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). The differences between control and medicine treated groups were analyzed using Student's *t*-test, and the *p* value of <0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

A. Characterization of the microfluidic device

To achieve uniform cell distribution and proper seeding density within a chip of microchambers is difficult, as the flow field within individual chambers is constantly changing as cells begin to aggregate during the seeding process. To address the cell seeding challenge, we designed the structure including U-shaped sieves within each cell chamber. Computational fluid dynamics simulations were carried out to optimize the cell chamber geometry.¹¹ The structure of the microfluidic chip [Figs. 1(a) and 1(b)] and single cell chamber with the optimized eight U-shaped sieves

was shown in Fig. 1(c). As depicted in Fig. 1(d), an adherent cell line MCF-7 was used to demonstrate the capability of this microfluidic device for cell culture and treatment. When cells were seeded into the cell chambers fabricated as the modeling result, cells were distributed uniformly with an average of 42 ± 3.1 cells within each chamber. After 24 h, culture cells in microchambers showed good adhesion and morphology, which was comparable to cells grown in 96-well plates.

B. MMC- and TAM-induced apoptosis in MCF-7 cells

MMC and TAM are well known (widely used) anticancer medicines. MMC is preferentially active compared to chemotherapeutic compounds with differing mechanisms of action such as cisplatin, docetaxel, or lovastatin.¹² TAM is a nonsteroidal triphenylethylene derivative and a selective estrogen receptor modulator, which has been used extensively in the treatment of both advanced and early-stage breast cancer.^{13–15} They have been reported to induce apoptosis of a variety of cancer cells.^{16–18} During the early stage of apoptosis, cells begin to display PS on cell surface membranes where it is readily detectable by staining the cells with Annexin-V-FITC.¹⁹ As the plasma membrane becomes increasingly permeable during the later stages of apoptosis, PI can move across the cell membrane and bind to DNA. Hoechst 33258 is a cell permeable nucleic acid dye, usually used to identify the total cells.

We first assessed MMC- or TAM-induced apoptosis of MCF-7, a breast cancer cell line, in microfluidic devices. Figure 2(a) showed the representative composite images acquired from a multiparametric cell apoptosis assay used to profile toxicity in MCF-7 cells. The correlative analysis of the apoptotic events associated with dose-response experiments was shown in Figs. 2(b) and 2(c). Early apoptotic cells or dead cells were only weakly detectable in medium treated chamber, the presence of MMC or TAM significantly induced apoptosis in MCF-7 cells. MMC caused a significant increase in the percentage of apoptotic cells compared with the control in a dose-dependent manner. Interestingly, TAM response peaks at 10 μM for MCF-7 cells in terms of magnitude, while TAM at the higher dose (50 μM) did not further increase the percentage of apoptotic cells as compared with TAM at 10 μM .

The cytotoxic effect is usually measured in 96-well plates using MMT assay. Therefore, as a comparison, we next assessed the cytotoxic effect of MMC or TAM in MCF-7 cell in a 96-well plate [Figs. 3(a) and 3(b)]. The results illustrated that both MMC and TAM dose-dependent reduced the viability of MCF-7 cells in 96-well plates, which was comparable with the observation in the microfluidic device. However, it was also notable that 50 μM of TAM, which most potently induced cell death in 96-well plates, did not further increase cell apoptosis and death as compared with 10 μM of TAM in the microfluidic device. This conflict might be due to the different fluid dynamics between the microfluidic device-based cell culture and the conventional cell culture in 96-well plates. As we have known, the microfluidic device-based culture system is characterized by continuous feeding of nutrients or drug to cultured cells and waste removal in the microfluidic device. In contrast, the 96-well culture is a completely static system. Therefore, in order to further define the effect of fluid dynamics on TAM-induced apoptosis, we shut down the outlets of the microfluidic device so that the cells were cultured in a static environment. The results [Figs. 2(d) and 2(e)] showed that the treatments with 2–50 μM of TAM for 24 h significantly increased the percentage of apoptotic cells in a dose-dependent manner compared with control. Notably, 50 μM of TAM most potently induced cell death in the static microfluidic device, which was comparable with the observation in a 96-well plate culture. Since two microfluidic device-based culture systems were similar except fluidic dynamics, the different results could be mostly due to the different fluidic dynamics. It has been shown that the continuous perfusion in microfluidic device might better mimic a wide range of physiological flow conditions than a static culture in traditional culture plates.²⁰ Therefore, it is necessary to further investigate the effects of the fluid dynamics on the cell based assays and compare the results obtained in microfluidic devices with the ones using traditional methods in the future.

FIG. 2. The cytotoxic effect of MMC and TAM on MCF-7 cells in microfluidic devices. MCF-7 cells were cultured and treated in microfluidic devices as described in Sec. II. (a) Representative phase contrast images and composite images from a multiparametric cell response used to profile cytotoxicity. Phosphatidylserine (Annexin-V-FITC, green), membrane permeability (PI, red), and nuclear morphology (Hoechst 33258, blue) were monitored using fluorescent staining. Magnification is $\times 20$. Scale bar is $100\ \mu\text{m}$. Correlative analysis of multiple apoptotic events associated with dose-response experiments under continuous flow [(b) and (c)] or static situation [(d) and (e)] in the microfluidic device. Counting the number of Hoechst 33258 positive cells, Annexin-V-FITC positive cells, and PI positive cells for each chamber, Hoechst 33258 positive cells were defined as the total number. Data were expressed as mean $\pm$ standard deviation (SD), the differences between control and treated groups were analyzed using the Student's t-test. Ψ : $p < 0.05$; *: $p < 0.01$; #: $p < 0.001$.

FIG. 3. The cytotoxic effect of MMC and TAM on MCF-7 cells in 96-well plates. 10^4 MCF-7 cells were precultured for 24 h and subsequently exposed with various doses of (a) MMC and (b) TAM for 24 h. The cell viability was measured using MTT assay, and the optical density was measured at a wavelength of 550 nm. Cell viability was calculated by using the following formula: (absorbance of drug-treated cells/absorbance of control cells) $\times 100$. Data were expressed as mean $\pm$ standard deviation (SD), the differences between control and treated groups were analyzed using the Student's t-test. Ψ : $p < 0.05$; *: $p < 0.01$; #: $p < 0.001$.

C. The expression of Bcl-2 induced by MMC and TAM in MCF-7 cells

Antiapoptotic protein Bcl-2 inhibits the release of cytochrome C from mitochondria and prevents the activation of caspase-9 and apoptosis in cancer cells.^{21,22} Several studies indicate that overexpression of Bcl-2 inhibits apoptosis induced by anticancer drugs, radiation, and other DNA-damaging agents.²³⁻²⁵ Pirnia *et al.*¹² found that MMC-induced apoptosis in the MCF-7 cells was associated with depletion of Bcl-2 and was independent of caspase-9 activation. Salami *et al.*

FIG. 4. The expression of Bcl-2 induced by MMC and TAM in MCF-7 cells. MCF-7 cells were cultured and treated in microfluidic devices as described in Sec. II. MCF-7 cells were incubated with FITC-labeled anti-Bcl-2 to detect Bcl-2 expression (green). The expression of Bcl-2 was determined by average optical density in each chamber. The average optical density was measured using IMAGE-PRO software. Data were expressed as mean $\pm$ standard deviation (SD), the differences between control and treated groups were analyzed using the Student's t-test. Ψ : $p < 0.05$; *: $p < 0.01$; #: $p < 0.001$.

FIG. 5. MMC and TAM inhibit the proliferation of MCF-7 cells. MCF-7 cells were cultured and treated in microfluidic devices [(a) and (b)] or in chamber sliders (c) as described in Sec. II. (a) Representative composite images associated with parameters of proliferation cells (anti-Ki-67-FITC positive, green) and nuclear morphology (Hoechst 33258 positive, blue) in MCF-7 cells. Magnification is $\times 20$. Scale bar is $100\ \mu\text{m}$. [(b) and (c)] Proliferation cells (anti-Ki-67-FITC positive) of each group (culture medium, treated by MMC or TAM) were numerated and the untreated group was defined as the total number. The percentage of cell proliferation was determined as per the formula (number of drug-treated proliferation cells/number of control proliferation cells) $\times 100$ and compared with control, untreated cells regarded as 100%. Data were expressed as mean $\pm$ standard deviation (SD), the differences between control and treated groups were analyzed using the Student's *t*-test. Ψ : $p < 0.05$; *: $p < 0.01$; #: $p < 0.001$.

reported that the expression of Bcl-2 did not change significantly with time on TAM.²⁶ Therefore, we evaluated the effect of MMC and TAM on Bcl-2 expression using immunofluorescent staining (Fig. 4). The results showed that MMC revealed a significant induction of Bcl-2 activity by depleting the Bcl-2 expression in a dose-dependent manner. The Bcl-2 protein level in MCF-7 cells at 24 h treatment with MMC at $50\ \mu\text{M}$ was reduced by about 58% of the control level. However, in contrast to MMC, there were no marked alterations in the levels of Bcl-2 protein in the MCF-7 cells treated by TAM. On average, the expression of Bcl-2 was unchanged relative to

the control value during TAM-treatment. Similar data were observed in chamber slides (data not shown). These results indicated that Bcl-2 protein might not be involved in TAM-induced apoptosis of MCF-7; however, Bcl-2 was one of a family of interacting proteins which influenced apoptosis and we did not investigate other members of that family. Therefore, MMC and TAM might induce the apoptosis of MCF-7 by different mechanisms.

D. MMC and TAM inhibited the proliferation of MCF-7 cells

The nuclear antigen Ki-67 is a proliferation marker expressed only in cycling cells. A strong correlation between S-phase fraction and Ki-67 index has been demonstrated.²⁷ The conventional measurements of the expression of Ki-67 in cells mostly include gel electrophoresis, immunoprecipitation, and western blotting.²⁸ The microfluidic device requires small volumes of reagents and samples than the existing macroscale technologies.²⁹ MMC or TAM caused inhibition rather than regression, they induced a significant apoptosis associated with inhibition of growth in cancer cells.³⁰

In the present study, we evaluated the expression of Ki-67 in MCF-7 cells cultured in the microfluidic device. Figures 5(a) and 5(b) showed that the growth of MCF-7 cells was inhibited by MMC or TAM in a concentration-dependent manner. The percentage of cell proliferation versus MMC was higher than TAM at 2 μ M indicating decrease susceptibility. MMC resulted in the lowest level of Ki-67 expression at 50 μ M and decreased the percentage of cell proliferation about 70% compared with the level for the control group. However, TAM at higher dose (50 μ M) did not further inhibit the proliferation as compared with TAM at 10 μ M. For comparison, we also evaluated the expression of Ki-67 in MCF-7 cells cultured in chamber slides. Figure 5(c) showed that the percentage of cell proliferation mediated by MMC or TAM was observed in a dose-dependent decline versus control using conventional immunofluorescent staining. The proliferation-inhibitory effect of MMC or TAM in MCF-7 cells in the microfluidic device was comparable to the data obtained from chamber slides.

IV. CONCLUSION

In this paper, we have developed a microfluidic device for apoptosis and proliferation analysis assays. The results in the microfluidic device were correlated well with the data obtained from the parallel experiments carried out in the conventional culture plates. The integrated microfluidic device is able to perform multiparametric pharmacological profiling with easy operation, thus holds great potential for extrapolation to the cell-based high content drug screening.

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