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Characterization of cell-type specific drug transport and resistance of breast cancers using tumor-microenvironment-on-chip

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Abstract

Heterogeneous response and resistance of cancer cells to chemotherapeutic drugs pose a significant challenge for successful cancer treatments. In this study, an integrated experimental and theoretical analysis of cellular drug transport was developed. The experimental platform, called tumor-microenvironment-on-chip (T-MOC), is a microfluidic platform where cancer cells were cultured within a three-dimensional extracellular matrix perfused with interstitial fluid. Three different human breast cancer cell lines (MCF-7, MDA-MB-231, and SUM-159PT) were cultured on this T-MOC platform, and their drug response and resistance to doxorubicin were characterized by time-lapse microscopy. To study the effects of nanoparticle-mediated drug delivery, the transport and action of doxorubicin encapsulated nanoparticles were also examined. Based on the experimental data obtained, a theoretical model was developed to quantify and ultimately predict the cellular transport processes of drugs cell-type specifically. The results demonstrate that the cellular drug transport can be cell-type specifically quantified by rate constants representing the uptake and efflux processes across the cellular membrane of doxorubicin.

INTRODUCTION

Heterogeneous response and resistance to chemotherapeutic drugs are one of the most significant clinical challenges for successful cancer treatments, and the realization of personalized or precision medicines. This is caused by tumor heterogeneity by genetic mutation^{1, 2} and the acquisition of drug resistance by various mechanisms.³ For example, triple-negative breast cancer (TNBC) is a significant clinical challenge due to its poor prognosis, which is associated with highly heterogeneous drug response and resistance.^{4–7}

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Supporting Information

Plot of fluorescence intensity as a function of drug concentration, bright field images presenting cell dependent morphologies and growth rates on TMOC with time, and time-lapse microscopy of breast cancer cells during drug treatment illustrated by using pseudo color. This material is available free of charge via the Internet at <http://pubs.acs.org>.

TNBC is a type of aggressive breast cancer, which does not express the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. Lehmann et al.⁸ recently identified six TNBC subtypes based on gene expression profiles and illustrated their highly heterogeneous drug response.

Moreover, it is further compounded with the complexity of tumor microenvironment. Besides multiple subpopulations of cancerous cells, various stromal cells including cancer associated fibroblasts and immune cells are present in the tumor microenvironment.^{9, 10} In addition to the heterogeneous biological composition, dense stroma and abnormal vasculature result in increased interstitial fluid pressure,^{11, 12} poor tissue perfusion, compromised nutrient and chemotherapeutic delivery,¹³ and hindered intratumoral penetration by drug macromolecules.¹⁴ These emergent properties of the complex, three-dimensional tumor microenvironment are characterized by spatiotemporally heterogeneous and transient cellular responses to therapeutic agents, posing significant challenges to effective treatment.¹⁵

Thus, an improved understanding of the dynamic response of cancer cells in physiologically appropriate environments will significantly accelerate drug discovery and improve treatment planning. To achieve this, new methods capable of providing detailed information of tumor cell responses during therapeutic treatment are highly desired. Such methods will enable elucidating mechanisms of chemoresistance and quantifying the extent of drug efficacy.^{15, 16} In this context, conventional two-dimensional cell cultures followed by a viability assay at an arbitrary time point are not adequate to provide a physiologically relevant understanding of the dynamic cell response. Although small animal models are widely utilized as a more physiologically complex chemotherapeutic screening platform, they typically are only able to provide an end-point evaluation without permitting detailed temporal insights into the tumor cell behavior throughout drug treatment.

Thanks to recent advances in tissue engineering and microfluidics, several *in vitro* models capable of recapitulating physical characteristics of the *in vivo* tumor microenvironment, while still permitting detailed investigation into tumor cell behavior have been proposed.¹⁷ Huang et al, developed a microfluidic co-culture construct in which different cell lines could be embedded and cultured in adjacent gels with different matrix substrates, establishing a model to study phenotypical changes induced by culturing tumor cells next to macrophages.¹⁸ Albanese and colleagues utilized a bioreactor platform to analyze early nanoparticle accumulation in tumor spheroids.¹⁹ Recently, a new platform has been developed called the tumor-microenvironment-on-chip (T-MOC) to mimic the complex pathophysiological transport within the tumor and surrounding microenvironment. In this microfluidic system, tumor cells and endothelial cells are cultured within a three-dimensional extracellular matrix (ECM) and perfused by interstitial fluid.²⁰ The T-MOC system is able to precisely modulate environmental parameters such as interstitial fluid pressure and tissue microstructure to analyze the significant effects each parameter dictates on nanoparticle and drug transport.

In this study, we developed an integrated experimental and theoretical analysis of cellular drug transport of breast cancers using T-MOC platform. Three different human breast cancer

cell lines (MCF-7, MDA-MB-231, and SUM-159PT) were cultured on this T-MOC platform, and their drug response and resistance to doxorubicin were characterized. To study the effects of nanoparticle-mediated drug delivery, the transport and action of doxorubicin encapsulated nanoparticles were also examined. Based on the experimental data obtained, a theoretical model was developed to quantify and ultimately predict the cellular transport processes of drugs cell-type specifically. The results were discussed to highlight the capabilities and limitations of the developed integrated model to achieve accelerated discovery of drugs and drug delivery systems and ultimately precision medicines.

MATERIALS AND METHODS

Cells and Reagents

Three types of human breast cancer cell lines (MCF-7, MDA-MB-231, and SUM-159PT) were used in this study. MCF-7 cells were maintained in a culture medium (DMEM/F12, Invitrogen) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/mL penicillin/streptomycin. The culture medium for MDA-MB-231 cells was supplemented with 10% FBS. SUM-159PT cells, obtained from Asterand (Detroit, MI), were cultured in a medium (Ham's F-12, Invitrogen) supplemented with 5% FBS, 10mM HEPES, 5µg/ml insulin, and 1µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO). All cells were cultured in 75 cm² T-flask at 37 °C and 5% CO₂. Cells were harvested for further experiments using 0.05% trypsin and 0.53 mM EDTA when cells reached 70~80% confluence.

Microfabrication of Tumor Microenvironment on Chip (T-MOC) Platform

The T-MOC platform is a two-layered microfluidic channels to mimic tumor vasculature, interstitium and lymphatics as illustrated in Figure 1. This configuration is designed to recapitulate tumor microenvironment consisting of tumor tissue sandwiched between a pair of capillary and lymphatic vessels, rather than a whole solid tumor. Details of the T-MOC fabrication and preparation were previously described.^{20, 21} Briefly, two different microchannel configurations were fabricated with polydimethylsiloxane (PDMS) by a standard photolithography technique. The top layer had a 300 µm wide single channel simulating a capillary vessel, and was connected to the bottom layer through an nano-porous membrane with 400nm pores (Cyclopore, Whatman) to enact leaky tumor capillary endothelium with impaired barrier function.^{14, 15} The bottom layer had three compartments mimicking tumor interstitium (middle channel with 900 µm wide) and lymphatics (two side channels with 300 µm wide). In order to assemble these channels, the membrane was treated with 5% 3-aminopropyl- triethoxysilane (Sigma-Aldrich, St. Louis, MO) solution and bonded with the PDMS layers after oxygen plasma treatment. After bonding, all channels were thoroughly washed with distilled water for 6 hours prior to loading cells. This channel configuration can mimic drug transport processes at tumor microenvironment including extravasation, interstitial transport and cellular uptake.²⁰

Cell Culture on the T-MOC Platform

After harvesting, the cell suspension was mixed with a solution of high concentration type 1 rat tail collagen (BD Biosciences, Bedford, MA) as described previously.²² The final

collagen concentration was 6mg/ml, and the final cell concentrations were 1×10^7 cells/ml for MCF-7 and MDA-MB-231, and 2×10^6 cells/ml for SUM-159PT. The lower cell concentration of SUM-159PT was chosen to alleviate cell mediated collagen compaction.^{23, 24} Then, the cancer cell-laden collagen solution was loaded to the T-MOC device. The T-MOC was incubated for 1 hour at 37 °C and 5% CO₂ for polymerization of collagen. After polymerization, the culture medium was perfused throughout the interstitial channel by controlling the pressure differences in the lymphatic and capillary channels as described previously.^{20, 21} Cells were cultured for two more days in order to obtain a representative 3D cellular microenvironment with cell-cell and cell-matrix adhesions before they were treated with drugs. During this pre-treatment culture, cells grew into multicellular clusters with tight cell-cell and cell-matrix adhesions as shown in the Supporting Information (FigureS5).

Time-lapse Microscopy during Drug Treatment and Post-treatment Viability Assay

As illustrated in Figure 2, after two days pre-culture, cells on the T-MOC were treated with doxorubicin. Two forms of doxorubicin were used in the study - 1) free doxorubicin hydrochloride (Dox-HCl, Sigma-Aldrich, St. Louis, MO); and 2) Dox-loaded 250nm hyaluronic acid nanoparticles (Dox-HANP), provided by the Therangnostic Research Group of Korea Institute of Science and Technology. Preparation of Dox-HANP can be found elsewhere.^{25, 26}

In order to test whether the T-MOC is capable of screening drug transport and action cell-type specifically, three breast cancer cell lines including SUM-159PT cell line was used to study the transport of free Dox. For the Dox-HANP transport, we used two lines – MCF-7 and MDA-MB-231 because of their clear difference in CD44 expression level, which is targeted by HANP.

After two-day of pre-treatment culture, the T-MOC was placed in a stage-top incubator (Okolab, Italy) under an inverted fluorescent microscopy. Each channel of the TMOC platform was pressurized to mimic elevated interstitial fluid pressure of tumor tissues - capillary fluid pressure (CFP) and interstitial fluid pressure (IFP) were approximately 20mmHg, and lymphatic fluid pressure (LFP) was 5 mmHg. At this pressure setting, the interstitial fluid flow was estimated to approximately 1 μ m/s. Then, either Dox or Dox-HANP pre-mixed medium at 2 μ M doxorubicin concentration was perfused along the capillary channel relevant to drug transport to tumor *in vivo*. In order to mimic *in vivo* pharmacokinetics of Dox or Dox-HANP (i.e., time-dependent clearance from blood stream), the drug-containing medium was perfused for 1, 3 and 24 hours.²⁷ For 1 and 3-hour treatments, after given drug exposure duration, the T-MOC was perfused with drug-free medium until 24 hour time point. The transport and accumulation of Dox and Dox-HANP was quantified by time-lapse fluorescence images acquired every 2 hours for 24 hours. The imaging conditions were controlled to be identical for all experiments to obtain the reliable drug concentrations from the acquired fluorescence images. The exposure time to the excitation light source was remained at 2000 ms for capturing a single image. Time-lapse images were taken every 1 or 2 hours to minimize the possible effects of phototoxicity on cells and photobleaching of doxorubicin. At the same optical condition, the fluorescence

intensity without doxorubicin was quantified and it was subtracted from all images obtained during drug perfusion experiments. Z-stacked confocal images were taken in 20 μm interval, and images with a focal plan 20 μm below the membrane were used to quantify the spatiotemporal fluorescence intensity. After 24 hours drug treatments, the TMOC was carefully removed from the incubation stage and cells were cultured for another 24 hours with drug-free culture medium. This post-treatment culture was implemented to ensure enough time for the action of doxorubicin on cancer cells, which could be confirmed by cell death.

After 24 hour post-treatment culture, cell viability was assessed by evaluating membrane integrity using Hoechst 33342/propidium iodide (Sigma-Aldrich, St. Louis, MO). Survival fraction was determined by normalizing the alive cell nucleus area of drug treated group by that of the control group as below.

$$\text{Survival Fraction} = \frac{[\text{Nucleus Area of Live Cells}]_{\text{treatment}}}{[\text{Nucleus Area of Live Cells}]_{\text{control}}} \quad (1)$$

To delineate the effects of 3D perfused microenvironment, drug response of conventional 2D monolayer was also assessed. Both MCF-7 and MDA-MB-231 were cultured in 24-well plates, then treated with 2 μM Dox and Dox-HANP for 24 hours followed by a 24 hour post-treatment culture in drug-free culture medium. The same viability assay was performed as described.

Statistical Analysis

The statistical significant differences ($p < 0.05$) between groups were evaluated using the one- way ANOVA followed by a student t -test to compare differences between pairs of groups. All experiments were repeated at least three times and the data were reported in the form: mean $\pm$ standard estimated errors.

Theoretical Analysis of Cellular Drug Transport

A theoretical model to analyze cellular level drug transport was developed as illustrated in Figure 3. First, the time-lapsed micrographs during drug treatment were further processed to quantify the drug concentration at extracellular (C_{ex}) and intracellular spaces (C_{cell}). Since doxorubicin concentration is proportional to the fluorescence intensity, the concentration of both spaces were determined using a pre-determined correlation between the fluorescence intensity and doxorubicin concentrations. This correlation was established by imaging collagen gels with different concentrations of Dox and Dox-HANP. The interstitial space, i.e., extracellular space, was defined as the vicinity around the cell area three pixels (approximately 2.2 μm) away from the cell aggregates' boundary by use of a trainable Weka segmentation macro of ImageJ. All data were manually double checked to clearly delineate both regions of interest. Then, the values of C_{ex} and C_{cell} were determined by spatially averaging doxorubicin concentration respectively at each time step. For each experimental group, five cell aggregates with comparable size (approximately 50 μm as shown in Figure 3(a)) and comparable distance from capillary channel were selected to determine the mean

C_{cell} and C_{ex} to minimize possible variability induced by the size and location of aggregates. The volume and surface area of each aggregate were approximated to those of spheres.

Then, as shown in Figure 3 (b), the rate of intracellular doxorubicin concentration change can be modeled by balancing drug influx (J_{in}) and effluent (J_{out}) as follows:

$$\frac{d}{dt}(C_{cell} \cdot V_{cell}) = J_{in} - J_{out} \quad (2)$$

The influx of doxorubicin (J_{in}) is approximated to a carrier-mediated binding reaction. Doxorubicin can transport across the cellular membrane via both passive diffusion and a carrier mediated trans-membrane transport. However, the later is a more dominant process, because doxorubicin molecules have the innate ability to self-associate into dimers, which are not permeable to the cells lipid-bilayer membrane.^{28–31} Therefore, the uptake of Dox and Dox-HANP is governed by binding to carrier proteins specific to Dox and Dox-HANP respectively. On the contrary, the effluent term (J_{out}) can attribute to both unbinding from the carrier proteins, and efflux from the intracellular space by multi-drug resistance associated efflux pump. Considering all these mechanisms, these two flux terms can be expressed as below.

$$J_{in} = k_{on} A_{cell} C_{ex} \left(1 - \frac{C_b}{C_{max}}\right) \quad \text{and} \quad J_{out} = k_{off} A_{cell} C_{cell} \quad (3)$$

The influx (J_{in}) is modeled as a first order binding reaction with a reaction rate constant k_{on} . Since the reaction is also limited to available binding sites, the influx should be scaled with the fraction of available binding sites. This scaling factor is approximated to the ratio of the maximum possible amount of bounded drug (C_{max}) to the difference between the bounded drug amount (C_b) and C_{max} , which indicates available drug binding sites. The drug efflux (J_{out}) from cancer cell is also modeled as a reaction with reaction rate constant k_{off} . The surface area and volume of the cancer cell aggregates, denoted by A_{cell} and V_{cell} , were obtained from the experimental data as explained in Figure 3(a). In order to complete the model, we assumed that the bounded drug concentration at the boundary of cell aggregates (C_b) is proportional to the intracellular drug concentration (C_{cell}) at a given time as below.

$$C_b \approx f \cdot C_{cell} \quad (4)$$

The underlying rationales of this assumption are - i) the correlation between C_b and C_{cell} is governed by intracellular drug transport and metabolism; ii) these intracellular processes are cell-type specific; and iii) the drug concentrations are low enough so that these processes are independent of time. In order to apply this assumption and prevent any errors caused by cell apoptosis and auto-fluorescence of dead cell nuclei, only cell clumps alive throughout the experiment were analyzed. This was confirmed during the cell viability assay.

The drug concentration data (C_{cell} and C_{ex}) were collected for three breast cancer cell lines (MCF-7, MDA-MB-231, and SUM-159PT) and two drug delivery schemes (DOX-HCl and DOX-HANP). These concentrations were obtained by converting fluorescence intensity to local doxorubicin concentration. For this conversion, quantitative relationships between the fluorescence intensity and local doxorubicin concentration were experimentally established. While flowing known concentration of doxorubicin or Dox-HANP contained medium through the capillary channel, the fluorescence micrographs were taken through and without the membrane. After subtracting the background intensity, the fluorescence intensities were quantified and mean intensities were computed. Then, calibration curves were determined by curve-fitting these data as shown in Supporting Information Figure S1. The values of k_{on} , k_{off} and f/C_{max} were then estimated by fitting the solution of Equations (2–4) with the experimental data of C_{ex} to the experimentally observed transient C_{cell} profile using nonlinear regression program coded in MATLAB.

RESULTS

Time-Lapsed Microscopy of Cellular Drug Transport

Time-lapsed fluorescence microscopy of cellular transport of Dox is shown in Figure 4 (a). All the images were obtained when 2 μ M doxorubicin containing medium was perfused the T-MOC platform for 24 hours. The local fluorescence intensity is proportional to local doxorubicin concentration. For Dox form, highest drug accumulation was observed in MCF-7 cells compared to the other two triple negative breast cancer cell lines. In addition, some of MCF-7 clearly showed the loss of cell-cell adhesion with time and significantly higher fluorescence intensity indicating enormous drug absorption to cell nuclei, whereas most of MDA-MB-231 and SUM-159PT maintained tightly packed spheroid and lower intensity.

Also distinct morphological and motility changes were also noted as presented in Figure 4 (b). Generally, the morphology and motility of MDA-MB-231 were characterized by random translational motion of the cell during pre-treatment, followed by cell dilation, formation of apoptotic bodies, and rapid membrane shrinkage as the cells undergo apoptosis and cease motile behavior during drug treatment and post-treatment culture phases. Interestingly, MDA-MB-231 cells exhibit a response to the drug indicated by a sudden increase in motility followed by a sharp, sustained decline after about 6 hours of exposure to doxorubicin. The sharp increase in cell motility during drug treatment prevalent in MDA-MB-231 was not seen for MCF-7. Instead a relatively steady decline in motility was observed within the first two hours of drug treatment, and was sustained throughout culture. The evident contrast between the motile responses of MDA-MB-231 and MCF-7 cells indicates that even though the doxorubicin is reaching and killing both cell types through the same molecular mechanism, the rate at which the tumor cells undergo apoptosis and the motile dynamics of how cell death occurs is strikingly heterogeneous in the complex tumor microenvironment.

For Dox-HANP form, the drug accumulation on MDA-MB-231 cells significantly increased comparable to that on MCF-7 cells as shown in Figure 4 (c). This implies that DOX-HANP preferentially interacts with MDA-MB-231 cells than MCF-7 cells. This is thought to attribute to the affinity of hyaluronic acid (HA) to the CD44 receptor. The CD44 receptor is

overexpressed on various cancer cells, and recent studies show that tumors as well as stromal tissues of triple negative breast cancers have a high number of CD44 overexpressed cells including MDA-MB-231.^{32–35} This result is in accordance with the reported capability of HA nanoparticles which target CD44 overexpressed cancer cells.^{36, 37}

Cell-Type Specific Drug Response and Resistance

Survival rates of the cancer cells after drug treatments are shown in Figure 5. As noted in the fluorescence micrographs in Figure 5(a), most of MCF-7 cells on the T-MOC platform were dead (noted with pink color) while MDA-MB-231 and SUM-159PT cells can survive the same doxorubicin treatment. Compared to each control group, total number of cells in all treatment groups (noted with pink color) decreased after the drug treatments. This implies that doxorubicin impede cell growth of triple negative breast cancer cell lines (MDA-MB-231 and SUM-159PT) but failed to cause ultimate cell death as noted in MCF-7 group.

Corresponding survival fractions on the T-MOC platform are as shown in Figure 5(b), and compared with the survival fractions of 2D monolayer culture. Overall, the survival rates of the cells cultured on the T-MOC platform are higher than the cells in 2D monolayer. This increased drug resistance is thought to be caused by the transport barriers of the T-MOC platform mimicking blood flow-driven convection, extravasation, interstitial transport and cellular transport, which are not present in 2D monolayer configuration.²⁰

Although the difference in the survival rates between different cell types are statistically significant on the T-MOC platform, these differences are not observed on the cells cultured in 2D monolayer. As mentioned, the triple negative breast cancer cell lines show significantly higher drug resistance than MCF-7 cells when these cells were cultured and treated with doxorubicin on the T-MOC platforms. However, on 2D configuration, the survival fraction of MCF-7 cells is higher than that of MDA-MB-231 or is comparable to that of SUM-159PT. This altered drug resistance trend suggests that the T-MOC platform provide cellular microenvironments closer to the *in vivo* tumor microenvironment compared to 2D monolayer. The survival rates of the cells treated with Dox-HANP are much higher than those of Dox groups, even though fluorescence images taken during drug treatment clearly showed higher dox accumulation in tumor spheroids compared to Dox. This implies that transported Dox-HANPs to targeted tumor might not release sufficient doxorubicin in the intracellular space.

Decreasing Dox perfusion time from 24 to 1 hours resulted in higher survival fraction, and the effect of perfusion time was more significant in less malignant MCF-7 cells, as shown in Figure 5 (c), so that decreasing Dox perfusion from 24 to 3 hours increased survival by approximately 3 times in MCF-7.

Theoretical Analysis of Cellular Drug Transport

Theoretical analysis was performed to determine the cellular drug transport parameters in Equation (2), i.e., drug binding rate constant (k_{on}) and efflux rate constant (k_{off}) as shown in Figure 6. The temporal drug concentrations at both cellular and interstitial spaces were obtained by quantifying the fluorescence intensity of the time-lapsed micrographs acquired during the drug treatments. The temporal variation of cellular drug concentration (C_{cell}) was

curve-fitted from the interstitial drug concentrations (C_{ex}), shown as symbols in Figure 6. The curve-fitted cellular concentration is shown as solid line in Figure 6. Since the regression analysis is a numerical integration of Equation (2), the curve-fitted cellular concentration is not a smooth line. However, it matches with the experimental data of all experimental groups very well.

The computed two rate constants are shown in Figure 7. The doxorubicin binding rates of all three cell lines studied are similar and have no statistically significant difference. However, MDA-MB-231 showed significantly higher efflux rates compared to the other two cell lines ($p < 0.05$). Thus, high survival rates of MDA-MB-231 cells to doxorubicin treatment are thought to be caused by higher drug unbinding and efflux from the cells. On the contrary, high survival of SUM-159PT even with the similar drug binding and efflux rate to those of MCF-7 implies that this triple negative cell line have different drug resistance mechanisms.

Compared with Dox, Dox-HANP show significantly higher binding rates to MCF-7 and MDA-MB-231. Moreover, the increase of the Dox-HANP binding rate of MDA-MB-231 is statistically significantly higher than that of MCF-7. This is thought to be caused by the preferential binding of hyaluronic acid to CD44 over-expressed cancer cells such as MDA-MB-231.³⁶ It has been reported that MDA-MB-231 express significantly higher levels of CD44 expression than MCF-7 cells.⁴⁸ However, the Dox-HANPs have higher efflux rates as well. This may result from the increased size of Dox-HANP so that, although the binding to the cells increases, the particles tend to unbind more easily or are internalized less efficiently.

Prediction of Cellular Drug Transport

The estimated cellular drug transport parameters of Dox were further used to predict drug transport during different treatment durations. These experiments were designed to mimic the effects of *in-vivo* pharmacokinetics where drugs are cleared from the bloodstream rapidly.^{15, 38} In this study, the breast cancer cell lines on the T-MOC platform were exposed to the doxorubicin for 1 and 3 hours. The experimental results are shown in Figure 8 with the prediction of cellular drug concentration based on the model developed in Equations (2–4) using the parameter estimated in Figure 7. Regardless of the treatment duration, the predicted cell drug concentration matches well with the experimental data. This suggests that the estimated cellular drug transport parameters are independent of systemic pharmacokinetics but more cell-type specific parameters.

DISCUSSION

All cell lines studied exhibited distinct responses to identical drug treatments. The chemotherapeutic agent employed in this study, doxorubicin, is a widely used member of the anthracycline antitumor drug family. Anthracyclines have proven to exhibit anti-cancer effects across a vast array of cancer types.^{39, 40} Doxorubicin diffuses across the cell membrane and induces apoptosis by intercalating DNA and producing numerous intracellular cytotoxic effects including activating caspases,⁴¹ disturbing mitochondrial membrane potential, damaging DNA,⁴² and increasing production of toxic free radical oxygen species (ROS).⁴³ During apoptosis, the membrane blebs to form apoptotic bodies,⁴⁴

and the cytoskeleton is drastically altered due to caspase-mediated actin cleavage,⁴⁵ as well as actin and microfilament reorganization, radically modifying the appearance and motile behavior of the cell. While the precise mechanisms driving the heterogeneous tumor cell response are unclear and were not explored in this study, the presented analysis method captures insightful trends in the cell-type specific response and may serve as a platform from which to base investigations to link tumor chemosensitivity with the complicated transport mechanics and biochemical factors regulating the extracellular tumor microenvironment.

In addition to gene mutation and molecular drug resistance mechanisms, biophysical aspects of tumor microenvironment also contribute to the drug response and resistance. This is caused by transport barriers engendered by pathophysiological characteristics of tumors including leaky and chaotic vasculature of the tumor, elevated interstitial fluid pressure, dense ECM microstructure and high cell packing density.^{15, 38} These barriers hinder transport of drugs and drug delivery systems at the tumor microenvironment. Moreover, the presence of 3D ECM architecture and tumor cell adhesion to the ECM can also trigger cellular drug resistance mechanisms.^{46, 47} In order to accurately characterize drug response, all these biophysical factors should be considered as well as biological ones. In this context, the present experimental platform provides a highly relevant cellular microenvironment to test drug response of various cancer types, since it provides 3D extracellular environments under perfusion.

The present results suggest that doxorubicin effectively binds at the similar rates to all three breast cancer cell lines studied. Moreover, significant increase of binding to MDA-MB-231 cells could be achieved by CD44 targeted HANPs. Thus, targeting CD44 via HA for targeted drug delivery to TNBC tumor cells could be a very potent strategy to treat advanced TNBC. However, these effective binding of doxorubicin and increased binding of HANPs did not result in improved cell death. This can be explained with the differences in the drug efflux rates. The significant difference of doxorubicin transport was noted in the efflux rate constant k_{off} rather than the binding rate constant k_{on} . As noted in Figure 7, MDA-MB-231 cell lines showed significantly higher drug efflux rate than MCF-7 cell lines. This explains why MDA-MB-231 shows significantly higher survival rate after doxorubicin treatment than MCF-7 cells as shown in Figure 3. Interestingly, the survival rate of both cell lines in 2D culture show opposite trend. This difference between 2D culture and T-MOC platform implies that the T-MOC platform may be able to provide certain aspects of tumor microenvironment, which are missing in 2D culture. Although further research is warranted, the drug transport parameters determined in this study may be relevant to predict the drug transport *in vivo*. In addition, previous computational analysis results need to be carefully interpreted and used since many were developed with rate constants acquired by fitting monolayer drug treatment experiment.^{28–30}

Although the magnitude of both transport rate constants increases, the same trend was observed for HANPs. This suggests that, while designing targeted drug delivery systems, binding to targeted ligands, which are typically located at cellular membranes, is a necessity but is not a sufficiency for effective delivery of drugs. Therefore, to increase drug transport into intracellular space and ultimately improve therapeutic efficacy, reducing unbinding from carrier proteins or efflux from the intracellular space can be exploited. In spite of the

improved binding, the cells treated with HANPs show less cell death than doxorubicin treatment. These results could be caused by that internalized Dox-HANPs did not release sufficient doxorubicin in the intracellular space during the time window of internalization.

The developed experimental and theoretical model enables quantitative analysis of cellular drug transport and mechanistic explanation of drug resistance mechanisms, but it still needs to be further developed. As mentioned, the efflux rate constant k_{off} is a combined measure of drug unbinding and efflux processes. However, the current experimental platform and the model cannot distinguish these two processes. Moreover, further imaging techniques and theoretical model to analyze the intra-cellular drug metabolism is highly desired. Although the T-MOC platform provides a cellular microenvironment closer to the *in vivo* tumor microenvironment than 2D cell culture and spheroid models, the current platform is still limited to fully recapitulate *in vivo* tumor microenvironment. These limited aspects include lack of tumor-stromal interactions, hypoxia, and much higher cell packing density of *in vivo* tumor tissues. In the present T-MOC platform, barrier functions of capillary and lymphatic vessels were mimicked using porous membranes and controlling the cross-sectional area through which fluid drains from the interstitial channel. Since tumor vasculature does not have well-developed endothelium with tightly aligned endothelial cells, the present design may provide microenvironment to study transport of drugs and nanoparticles which are not directly targeting endothelial cells. However, lack of endothelial cells is not suitable to study transport and action of drugs and nanoparticles whose major action mechanisms are associated with endothelial cells.

CONCLUSION

An integrated experimental and theoretical model is developed to quantitatively characterize the drug response and resistance of TNBC cell-type specifically. The experimental model, T-MOC platform, is an *in vitro* tumor model capable of creating microenvironment mimicking the *in vivo* tumor microenvironment. The theoretical model demonstrates that the cellular drug transport can be cell-type specifically quantified by rate constants representing the uptake and efflux processes across the cellular membrane of doxorubicin and Dox-HANPs. Although further research is still warranted, the developed model will be useful to rapidly identify effective drug and drug combinations cell- and patient- specifically for drug discovery and ultimately personalized medicines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of the assembly process of the double-layers TMOC. The top PDMS layer (capillary channel), nanoporous membrane, and bottom PDMS layer (interstitial and lymphatic channel) were aligned and tightly bonded using oxygen plasma treatment. This fabricated double-layers TMOC was designed to establish the *in vitro* tumor model by simplifying physiological tumor environment (right).

Figure 2.

Timeline of drug treatment experiment and subsequent viability assay. After 2 days pre-culture on the T-MOC platform, cells were treated with doxorubicin for 1, 3, and 24 hours while the T-MOC was pressurized to mimic the elevated interstitial fluid pressure of tumor interstitium. The interstitial fluid pressure (IFP) was controlled at 20 mmHg, capillary fluid pressure (CFP) maintained at 20 mmHg, and lymphatic fluid pressure (LFP) was 5 mmHg respectively. After drug treatment, the T-MOC was perfused with drug-free culture medium for 1 day and then cell viability was assessed.

Figure 3.

Schematics of theoretical analysis of cellular drug transport. (a) Time-lapsed fluorescent micrograph of the cells during drug treatment was analyzed to determine drug concentrations at extracellular and intracellular spaces. The volume and surface area of the cell aggregates were computed by approximating to a sphere with the same cross-sectional area. (b) Cellular drug transport across the cell membrane was modeled considering drug binding, unbinding and efflux.

Figure 4.

Time-lapse microscopy of breast cancer cells during drug treatment. (a) Fluorescent micrographs during Dox treatment for 24 hours. (Scale bar = 50 μm) (b) Brightfield micrographs showing morphological and motility changes by Dox treatment. The size of arrows indicates the magnitude of cellular motility. Contrast to MCF-7, MDA-MB-231 cells show increased motility during Dox treatment. (c) Dox-HANP treatment for 24 hours. (Scale

bar = 50 μ m) All the fluorescence micrographs were pseudo-colored using pre-calibrated doxorubicin concentration (Figure S1).

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Figure 5.

Tumor viability assay after 1 day post-treatment culture. The levels of all and dead cells were distinguished by staining cell nuclei using Hoechst 33342 and propidium iodide. Blue and red indicate cell nuclei area for all cells and dead cells, respectively (a). The survival fractions of MCF-7, MDA-MB-231, and SUM-159PT caused by DOX-HCl and DOX-HANP treatment on 2D monolayer and TMOC (3D) were presented (b and c). Means sharing a symbol are significantly different (p -value < 0.05). Error bars represent standard deviation ($n=3$).

Figure 6.

Theoretical analysis of cellular drug transport during 24-hour treatment of Dox and Dox-HANP: (a) MCF-7, (b) MDA-MB-231, and (c) SUM-159PT. Experimentally observed drug concentrations in cell (C_{cell} , black square) and interstitium (C_{ex} , red triangle) are shown with the curve-fitted concentration (blue solid line) from the theoretical model developed. The experimental data were obtained from four repeated experiments ($n=4$). The concentrations were normalized by the maximum concentration in the cell for a given experiment.

Figure 7.

Cellular drug transport parameters of doxorubicin to breast cancer cell lines: Binding (k_{on}) and efflux (k_{off}) rates. (m/sec) The binding rates of Dox to all three cell lines are similar, but the efflux rates are cell-type dependent. The Dox-HANPs have significantly higher drug binding rates than Dox-HCl, and more efficiently bind to MDA-MB-231. However, the efflux rates are also increased. The values of k_{on} and k_{off} sharing the same symbol are significantly different ($p < 0.05$). These values were obtained from individual nonlinear regression of four experiments (n=4).

Figure 8.

Prediction of cellular drug transport during 1-hour and 3-hour treatment of Dox: (a) MCF-7, (b) MDA-MB-231, and (c) SUM-159PT. Experimentally observed drug concentrations in cell (C_{cell} , black square) and interstitium (C_{ex} , red triangle) are shown with the predicted concentration (blue solid line). The predicted concentrations were computed based on the theoretical model, i.e., Equation (2), with the experimentally measured interstitial drug concentration (C_{ex}) and the binding (k_{on}) and efflux (k_{off}) rates determined in Figure 7. The experimental data were obtained from four repeated experiments ($n=4$), and, in each experiment, at least five cell aggregates were analyzed. The concentrations were normalized by the maximum concentration in the cell for a given experiment.