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Microscale Mass Spectrometry Analysis of Extracellular Metabolites in Live Multicellular Tumor Spheroids

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Abstract

Extracellular compounds in tumors play critical roles in intercellular communication, tumor proliferation, and cancer cell metastasis. However, the lack of appropriate techniques leads to limited studies of extracellular metabolite. Here, we introduced a microscale collection device, the Micro-funnel, fabricated from biocompatible fused silica capillary. With a small probe size ($\sim 25 \mu\text{m}$), the Micro-funnel can be implanted into live multicellular tumor spheroids to accumulate the extracellular metabolites produced by cancer cells. Metabolites collected in the Micro-funnel device were then extracted by a microscale sampling and ionization device, the Single-probe, for real-time mass spectrometry (MS) analysis. We successfully detected the abundance change of anticancer drug irinotecan and its metabolites inside spheroids treated under a series of conditions. Moreover, we found that irinotecan treatment dramatically altered the composition of extracellular compounds. Specifically, we observed the increased abundances of a large number of lipids, which are potentially related to the drug resistance of cancer cells. This study provides a novel way to detect the extracellular compounds inside live spheroids, and the successful development of our technique can benefit the research in multiple areas, including the microenvironment inside live tissues, cell–cell communication, biomarker discovery, and drug development.

Graphical abstract

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Notes

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Lipids significantly regulated by irinotecan treatment, MS/MS analysis of irinotecan, irinotecan metabolites, and selected cell metabolites (PDF)

Cancer recurrence is one of the major threats for the health of patients receiving chemotherapy.^{1,2} For example, around 40% of patients with breast cancers can develop recurrent metastatic diseases after chemotherapy.^{3,4} Recent studies indicate that central tumor cells play crucial roles in cancer recurrence.^{5,6} Due to the distribution gradients of oxygen, nutrients, and energy, tumors possess gradients of proliferation cells and metabolites abundances from outer to inner regions.⁷⁻⁹ Generally, central tumor cells stop cell cycle progression and become dormant due to their microenvironment, while cells in the outer regions of tumors have high proliferative activities.⁵ Because cytostatic drugs mainly target proliferating cells, central cancer cells with low proliferating rate could survive from drug treatment and potentially cause the disease relapse after the therapy.^{5,6,10} Previous studies found that extracellular compounds are critical in multiple biological behaviors of tumors,^{11,12} including tumor growth and progression, cell-cell communication, and cancer cell migration and metastasis.^{13,14} Studying extracellular compounds produced by central tumor cells can potentially identify more specific cancer biomarkers, reveal the characteristic responses of cancer cell under drug treatment, and provide guidelines for the amelioration of current cancer therapies.

Conventionally, two-dimensional (2D) cell monolayers are widely used for drug discovery studies.¹⁵ However, results obtained from 2D cultured cells cannot be directly translated into in vivo settings because of their totally different microenvironment compared with cells in actual tissues. For example, the 2D cell monolayers are exposed to sufficient oxygen and nutrients, whereas the microenvironment of cells in solid tumors contains gradients of oxygen, energy, and some critical chemicals.¹⁶ Previous studies indicate that the extracellular compounds secreted by 2D-cultured cells are much less abundant than those in a three-dimensional (3D) environment, suggesting that interactions among cells in 2D models are different from those in a 3D environment.¹⁷ It is evident that, compared with 2D culture, 3D cell cultures can better mimic the microenvironment and cell-cell interactions of in vivo tumors.¹⁸ The multicellular tumor spheroids (hereinafter referred to as spheroids) are widely used 3D tumor models.^{19,20} Spheroids have been proven to possess numerous characteristics similar to in vivo tumors, such as extracellular compounds and protein production patterns,^{20,21} making them more accurate, ideal models for the research of extracellular molecules.

However, studies of broad ranges of molecules inside live tumors have not been widely carried out due to the lack of appropriate analytical techniques. In a long time, the detection

of extracellular biomolecules is limited by their low concentrations, especially once they diffuse into the cell culture medium or dilute in body fluid. Microdialysis devices, which use a semipermeable membrane to enrich molecules, have been utilized to study the extracellular compounds of tumors or tissues.^{22–24} However, the general sizes of microdialysis probes range between 200 and 500 μm , with the smallest one around 200 μm ,^{25–27} resulting in limited spatial resolution. Therefore, it is very challenging to use these techniques to study relatively small targets such as spheroids (sizes are generally less than 1 mm),^{19–21} tumors at early stages, or small regions inside large tissues. In addition, lipophilic compounds may be retained by the tubing or other probe components, resulting in a discrepancy of components sampling and low or no analyte recovery of the amount of sample solution needed.^{28,29}

In this study, we developed a microscale device, the Micro-funnel, to collect the extracellular molecules produced from cells in the central region of spheroids. The Micro-funnel has a small tip size ($\sim 25\ \mu\text{m}$), which allows it to be implanted into a live spheroid for direct collection of extracellular compounds without being diluted and selected. A Single-probe device is inserted into the orifice of the Micro-funnel to extract the collected extracellular molecules for direct mass spectrometry (MS) analysis. The Single-probe is a miniaturized multifunctional sampling and ionization device that can be coupled with MS for the real-time microscale bioanalysis. The Single-probe MS techniques have been successfully used to study live single cells (single cell MS) and to map biomolecules on animal tissues (MS imaging) with high spatial resolutions.^{30–34} Due to its extremely small sampling probe size ($<10\ \mu\text{m}$) and high efficiency of extraction and ionization, the Single-probe is an ideal device to analyze small amounts of biomolecules collected in the Micro-funnel device. By combining the Micro-funnel with the Single-probe, we measured anticancer drug irinotecan and its major metabolites in the central region of spheroids and determined their abundance changes under different treatment concentrations and time lengths. In addition, we investigated the influence of irinotecan treatment on the compositions of extracellular metabolites. Our study provides a new tool for the research of extracellular drug metabolites and cell metabolites in live spheroids.

EXPERIMENTAL SECTION

Fabricate the Single-Probe and the Micro-funnel

The detailed fabrication protocols of the Single-probe were provided in our previous work.^{30,34} Briefly, a Single-probe (Figure 1a) consists of three components: a dual-bore quartz tubing (outer diameter (OD) of 500 μm ; inner diameter (ID) of 127 μm , Friedrich & Dimmock, Inc., Millville, NJ, USA) pulled using a laser pipet puller (P-2000 micropipette puller, Sutter Instrument, Novato, CA, USA), a fused silica capillary (OD of 105 μm ; ID of 40 μm , Polymicro Technologies, Phoenix, AZ, USA), and a nano-ESI emitter produced using the same type of fused silica capillary. A Single-probe is fabricated by embedding a laser-pulled dual-bore quartz needle with a fused silica capillary and a nano-ESI emitter.^{30,34} The Micro-funnel (Figure 1b) is produced using a fused silica capillary (OD of 360 μm ; ID of 70 μm , Polymicro Technologies, Phoenix, AZ, USA). Upon removing the coating material, the fused silica capillary is pulled using the laser pipet puller (tip size of $\sim 25\ \mu\text{m}$) and cut into a Micro-funnel with a desired length ($\sim 5\ \text{mm}$).

Implant Micro-funnels in Spheroids

The colon carcinoma cell line HCT-116 was used as the model to culture spheroids. First, we cultured cells following the standard protocols. Cells were cultured in McCoy's 5A cell culture medium (Life Technologies, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum; Life Technologies, Grand Island, NY, USA) and 1% Pen Strep (Life Technologies, Grand Island, NY, USA). A CO₂ incubator (HeraCell, Heraeus, Germany) was used to culture cells under well-controlled conditions (5% CO₂, 37 °C), and cell passage was performed every 2 days. Second, we cultured spheroids using HCT-116 cells. The culture protocol of spheroids was generally adopted from previous studies with some modifications.^{35–37} To foster the accumulation of cells and promote the success rate of culturing spheroids, agarose-coated 96-well plates (with U-bottom wells) were utilized in our modified protocols. Briefly, 60 μ L of agarose gel (1.8%; Sigma-Aldrich, St. Louis, MO, USA) was added into individual wells in a 96-well plate (U-bottom; VWR, Radnor, PA, USA). 200 μ L of cell solution (containing ~12000 cells) was added into the agarose-coated wells, and the Micro-funnel was horizontally placed on the top of the agarose coating with its tip positioned in the well center. Because the fused silica is a biocompatible material,³⁸ cells incubated under normal conditions (5% CO₂, 37 °C) can attach to and grow around the Micro-funnel tip. Culture medium was changed every 48 h, and spheroids with implanted Micro-funnels were collected after 14 days of culture. Using an inverted microscope, we selected a similar size (diameters of ~1 mm) of spheroids, in which the Micro-funnels tips were implanted in the center regions. Eight ($n = 8$) samples were used in each group for drug treatment and control experiments. The screening process and replicated measurements allow us to minimize the influence of sample variation on experimental results.

Drug Treatment of Spheroids

Irinotecan was purchased from Life Technologies (Grand Island, NY, USA). The stock solution of irinotecan was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and further diluted into McCoy's 5A cell culture medium to prepare drug solutions with a series of concentrations. To perform drug treatment, a plastic microscope slide (VWR, Radnor, PA, USA) with a pinhole (diameter: ~360 μ m) was prepared and placed on the top well in the 96-well plate (Figure 1c). The pinhole on the plastic slide was used to vertically hold the Micro-funnel implanted into a spheroid. Irinotecan solutions were added into wells to submerge the spheroid, whereas the orifice of the Micro-funnel was retained above the treatment solution, eliminating any infusion of treatment solution into the Micro-funnels. We have conducted both concentration- and time-dependent studies. In the concentration-dependent experiments, spheroids were treated for 5 h using a series of concentrations (5, 20.6, 50, 100, 200, 300, 400, and 500 μ M). In time-dependent studies, we used the IC₅₀ of irinotecan (20.6 μ M)³⁹ and treated it for various lengths of time (5, 12, 24, and 48 h). Untreated spheroids were used as the control group in the experiments.

Couple the Micro-funnel with the Single-Probe MS Setup

The extracellular contents collected in the Micro-funnel are present in a very small volume (~1.2 nL) which is challenging for conventional MS analysis. The Single-probe device, which is designed for microscale MS analysis, is appropriate for analysis of molecules

collected in the Micro-funnel. To conduct an experiment, the 96-well plates containing spheroids were placed on a home-built *XYZ*-translation stage system (Figure 1d),^{30,34} and the spatial motion of the stage was controlled with the LabView software package.⁴⁰ Using the microscope as a visual guide, the tip of the Single-probe (OD ~ 10 μm) was precisely placed on the orifice of a Micro-funnel (ID ~ 70 μm); a small gap between the Single-probe tip and Micro-funnel orifice is present due to the size difference. The sampling solvent (acetonitrile; Sigma-Aldrich St. Louis, MO, USA) was continuously provided (~0.1 $\mu\text{L}/\text{min}$) by a syringe (250 μL ; Hamilton Co., Reno, NV, USA), and a liquid junction formed at the Single-probe tip performs a highly efficient extraction of extracellular contents collected inside the Micro-funnel. The extracted molecular species were withdrawn by the nano-ESI emitter through the capillary action and immediately ionized by a voltage applied on the conductive union followed by MS analysis using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). Mass analysis parameters are listed as follows: mass range (m/z), 200–1500; mass resolution, 60 000; ionization voltage at positive ion mode, +4 kV; microscan, 1; max injection time, 100 ms; AGC (automatic gain control), on.

Under the optimized conditions, stable ion signals can be obtained without having bubbles inside the emitter (i.e., solvent flow rate is too low) or solvent dripping on the Single-probe tip (i.e., solvent flow rate is too high). Due to the gap between the Single-probe tip and Micro-funnel orifice as well as the optimized flow rate, the sampling solvent is unlikely to be delivered into spheroids through the Micro-funnel affecting the cell growth environment. The solvent transport through diffusion is also negligible. A MS measurement of a spheroid is usually accomplished within 5 min, which is marginal compared to the time needed for acetonitrile molecules to diffuse through the Micro-funnel to reach the cells (~2.4 h). The diffusion time can be estimated using $t = x^2/2D$, in which x (5 mm) is the diffusion distance and D ($1.445 \times 10^{-5} \text{ cm}^2/\text{s}$) is the diffusion coefficient of acetonitrile in water.

Data Processing

The molecular identification of irinotecan and its metabolites was performed by comparing their accurate m/z values with theoretical results. More confident analyses were carried out by conducting tandem MS analysis (MS/MS or MS², i.e., fragments analysis of selected ions) and comparing with results from the standard irinotecan sample and published work.^{39,41} However, a real biological tissue consists of complex histological structures and chemical components, and a large amount of information is present within each mass spectrum. Manual analysis of MS data can only provide limited and selected information on the species among numerous amounts of data. In contrast, statistical analysis methods, including principle component analysis (PCA), t test, and analysis of variance (ANOVA), are powerful tools to extract features from complex MS data.^{42–45} We utilized statistical data analysis methods to analyze MS results obtained from spheroids experiments. First, we export peaks (with m/z values and relative intensities) into Excel data format using Thermo Xcalibur Qual Browser (Thermo Scientific, Waltham, MA, USA). Due to the extremely complexed MS data, we only export relatively abundant peaks (i.e., ion intensities higher than 10^4) for analysis; the background noise level is $<10^3$. To minimize the influence induced by fluctuations of ion signals during experiments, we used the relative ion

intensities normalized to the total ion current (TIC). Second, we upload the Excel data onto online software *Geena2* (<http://bioinformatics.hsanmartino.it/geena2/>)⁴⁶ for peak alignment. Third, we import treated data into *Metaboanalyst 3.0* (<http://www.metaboanalyst.ca/faces/ModuleView.xhtml>)^{47–55} to conduct statistical data analysis, including PCA and the *t*-test. PCA was utilized to detect the difference of chemical compositions between drug treated and control groups, whereas the *t* test was applied to extract characteristic molecular peaks with significant abundance changes ($P < 0.05$) upon drug treatment. Finally, we use online database METLIN (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage)⁵⁶ and human metabolome database (HMDB; <http://www.hmdb.ca>)^{57–59} to tentatively label all ions of interest, which can be grouped into a few different major categories using the heat maps. In addition, ANOVA was performed to further demonstrate that most species (299 out of 330) reported in heat maps and tables (Tables S1 and S2) were significantly altered by drug treatment as listed in Table S3.

RESULTS AND DISCUSSIONS

Detection of Irinotecan and Its Metabolites

Although the distribution of irinotecan and its metabolites in spheroids has recently been reported,^{39,60} the drug abundance change and the metabolites composition in the microenvironment inside a living tumor are still unclear. The combined Micro-funnel and Single-probe design aims to collect and detect anticancer drugs, drug metabolites, and cell metabolites inside live spheroids. As shown in Figure 2, using our techniques, we have successfully detected the drug compound and multiple drug metabolites, including SN-38 (m/z 393.1437), hydroxyl-irinotecan (m/z 603.2802), decarboxyl-irinotecan (m/z 543.2956), and dehydro-irinotecan (m/z 585.2697). MS/MS analysis has been performed to further confirm the structures of irinotecan and its metabolites accumulated by the Micro-funnel (Figure S1). Irinotecan and its metabolites were not detected in spheroidoids in the control group.

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy camptothecin) has been widely used in clinical treatment of colon cancer and lung cancer.⁶¹ The metabolic pathways of irinotecan have already been reported,^{39,62} as partially summarized in Figure 3. Irinotecan is a pro-drug that can be metabolized by carboxylesterases into the active compound SN-38, a cytotoxic metabolite that is 300- to 1000-fold more active than irinotecan.^{41,63–65} SN-38 can cause irreversible induced DNA damage by targeting *Topoisomerase I* and stabilizing the DNA cleavable complex, resulting in cancer cell death.^{66,67} The detection of irinotecan from extracellular contents inside spheroids is expected since the drug molecules can be delivered into inner regions through molecular diffusion.⁸ The drug metabolite SN-38 detected in the extracellular environment is likely from the secretion of cancer cells.⁶⁸ Previous studies demonstrated that SN-38 can be expelled by colon cancer cells through the efflux mechanism, which is directly related to their drug resistance capabilities.^{69,70} For example, P-glycoprotein (P-gp), an ATP-binding cassette transporter, has been considered as the major transporter of irinotecan and SN-38.^{7,69} Similarly, the multidrug resistance-associated protein was also identified as a transporter of irinotecan and its metabolites.⁷¹

Another interesting observation is that, for spheroids treated with irinotecan, the abundance of decarboxyl-irinotecan (m/z 543.2956) has a similar trend to that of irinotecan (Figure 4). Although decarboxylated species was regarded as one of the irinotecan metabolites³⁹ (Figure 3), we have detected this compound (with ~1% relative ion intensities compared with irinotecan) in irinotecan water solutions (i.e., 5, 20, 100, and 500 μM). It is plausible that decarboxyl-irinotecan is produced through the in-source fragmentation of irinotecan parent ion. We further performed two types of experiments to better represent the influence of matrix effect on the detection of irinotecan and its metabolites. (a) We used spheroids (control group) to prepare cell lysate solutions containing irinotecan with the above concentrations. (b) We added irinotecan solutions (with the above concentrations) in the syringe and inserted the Single-probe tip into the Micro-funnel implanted in the spheroids (control group) to conduct MS measurements. The relative ion intensities of decarboxyl-irinotecan are ~1% compared with irinotecan in both types of experiments. However, this ratio ranges between 3% and 20% in the spheroid experiments depending on the treatment conditions: higher concentration and longer treatment time lead to more abundant SN-38 (Figure 4). Therefore, it is very likely that the extracellular decarboxyl-irinotecan detected from spheroids is primarily from irinotecan metabolism.

Abundance Change of Extracellular Irinotecan and Its Metabolites in Concentration- and Time-Dependent Experiments

In addition to the detection of molecules of interest inside live spheroids, we have investigated the abundance change of irinotecan and its metabolites under different treatment conditions. We have conducted comprehensive studies of spheroids treated using a series of concentrations and different lengths of time. In concentration-dependent experiments, we treated spheroids ($n = 8$ for each treatment concentration and control group) with irinotecan using a series of concentrations (5, 20.6, 50, 100, 200, 300, 400, and 500 μM) for 5 h. We measured the relative abundances, which were normalized to the TIC, of extracellular irinotecan and its metabolites. As shown in Figure 4a, the ion intensities of irinotecan increase with higher drug concentrations for treatment. Hydroxyl-irinotecan and SN-38 started to appear inside spheroids at relatively higher treatment concentrations (i.e., 100 and 300 μM for hydroxyl-irinotecan and SN-38, respectively). All four major metabolites were detected from spheroids treated with 500 μM irinotecan.

For time-dependent measurements, the adopted drug concentration was 20.6 μM , which is the IC_{50} of irinotecan for HCT-116 spheroids reported from previous studies.^{39,72} We treated spheroids ($n = 8$ for each treatment time) with 20.6 μM irinotecan for various lengths of time (5, 12, 24, 48, and 72 h). However, the treatment of 72 h resulted in a complete detachment of spheroids from Micro-funnels, and the corresponding MS measurements were not conducted. In general, abundances of irinotecan and its metabolites as well as the number of drug metabolites increase along with longer treatment time (Figure 4b). For example, the hydroxyl-irinotecan and SN-38 were detected after 12 and 48 h, respectively; however, they were not observed from samples with shorter time drug treatments.

The combined Micro-funnel and Single-probe device has been proven to successfully detect the extracellular drug and its metabolites in the central regions of spheroids treated under a

variety of different conditions. Our techniques can be used to study the pharmacokinetics of drugs (such as the drug absorption and excretion) and the rate of drug metabolism inside live tumors. These techniques can potentially provide an effective approach to investigate the fundamentals of antidrug ability of tumor cells and facilitate the determination of appropriate drug concentration needed in drug screening studies.

Influence of Drug Treatment on the Extracellular Metabolites Inside Spheroids

The molecular compositions of cell metabolites (including both intracellular and extracellular species) can rapidly respond to the changes of cell status and the living environment. Previous analyses of cell metabolites from tissues are primarily conducted using cell lysate prepared from tissues. The corresponding experimental results cannot discriminate between intracellular and extracellular metabolites of living tissues. This previously intractable task can be accomplished using the combined Micro-funnel and Single-probe device. Due to the critical roles of extracellular compounds on cell–cell communication and cancer cell migration and metastasis, we applied our techniques to investigate whether drug treatment can modulate the composition of extracellular compounds inside spheroids.

First, we demonstrated that compounds in culture medium have no interference with the analysis of molecules collected in the Micro-funnels, although our experiments were designed to prohibit the infusion of culture medium into the Micro-funnel during the drug treatment (Figure 1d). We conducted PCA of mass spectra obtained from three medium groups (i.e., the fresh medium, medium collected after spheroids culture, and the medium collected after 50 μ M irinotecan treatment) as well as samples collected in Micro-funnels from irinotecan-treated spheroids and the control group (Figure 5a). PCA results indicate that the molecular compositions in culture media are significantly different from those extracellular compounds inside spheroids. In fact, culture medium is composed of inorganic salts, amino acids, vitamins, and glucose. In contrast, the major metabolites detected in tumor environments include phospholipids, glycerides, fatty acids, and glycoside deviates (please refer to Figure 6 and Tables S1 and S2 for details). These results demonstrate that the Micro-funnel device can effectively accumulate extracellular compounds in center regions of spheroids without having the leakage of culture medium.

Second, we investigated the general trends of composition change of extracellular compounds induced by drug treatment under different conditions. PCA results show that samples from different treatment concentrations (Figure 5b) and time lengths (Figure 5c) exhibit different metabolic signatures. Moreover, the data points are generally located farther away from the control groups, and their distributions are broader for samples with higher concentrations (Figure 5b) or longer time (Figure 5c) for drug treatment. It is very likely that these trends are attributed to the metabolites change as cellular drug uptake increases, which is induced by higher drug concentration or longer time for treatment. Farther distance (i.e., higher scores on PCA plots) is due to the increased abundance of extracellular metabolites; broader distribution is likely associated with sample diversity (e.g., such as minor differences in size of spheroids and location of Micro-funnel implant), and the caused variations in cell metabolite become more significant as the drug uptake increases.

Third, for a better understanding of cell metabolic regulation and the response of tumor cells to drug treatment, we classified the extracellular metabolites that are significantly altered by anticancer drug treatment based on *t* test results. We tentatively assigned the extracellular compounds collected in Micro-funnels by searching the measured accurate *m/z* values in the METLIN and HMDB databases (mass accuracy, <5 ppm). Because previous studies found that drug treatment affects the metabolism of the extracellular lipids,⁷³ we focused our current study on analyzing the irinotecan-induced extracellular lipid change. As summarized in the heat maps (Figure 6a,b), the patterns of extracellular lipids are different between control and irinotecan-treated spheroids, and their compositions also change with the increase of drug concentration and length of treatment time. Specifically, lipids significantly changed by irinotecan treatment include phospholipids (phosphatidic acids (PAs), phosphoethanolamines (PEs), phosphatidylglycerols (PGs), phosphatidylserines (PSs), and phosphatidylinositols (PIs)) and glycerolipids (diglycerides (DGs) and triglycerides (TGs)). Although it is impractical to obtain the exact structure information on all tentatively assigned peaks, we can confidently classify these species into different groups such as PA, PE, PG, PS, PI, DG, and TG. In addition, our technology allows us to conduct MS² analysis of ions of interest for more confident structure identification. For example, four compounds (i.e., DG (38:5), PC (34:5), PC (40:7), and TG (50:3)) have been confirmed by performing MS² analysis (Figure S2) and comparing our mass spectra with the METLIN database. MS³ and the above analyses were not carried out due to inadequate ion intensities. The lists of tentatively labeled major species are provided in Tables S1 and S2. ANOVA was utilized to analyze species listed in Tables S1 and S2 to demonstrate differences between drug treated and control groups.

Previous studies found that some colorectal cancer cells can develop resistance to the SN-38 through regulating their cellular metabolism.^{74,75} For example, under irinotecan treatment, colon cancer cells can enhance de novo lipogenesis to accumulate cellular TGs and phospholipids, which can promote cell survival^{76,77} and develop the resistance to chemotherapy.^{78,79} TGs are used by cells for energy storage; phospholipids are essential components for the biological membrane, and they are also found as second messengers for transduction of intercellular signals.^{80–82} Both TGs and phospholipids are critical for cell proliferation and apoptosis.^{83–85} The enhanced lipogenesis in drug-treated cells can increase lipids secretion and result in increased concentrations of extracellular lipids.⁷⁹

Correspondingly, in our live spheroids studies, we did observe the increase of extracellular lipids (e.g., phospholipids, TGs, and DGs) under irinotecan treatment (Figure 6a,b), which was likely attributed to the triggered drug resistance of cancer cells inside central regions of tumors. A full list of grouped species shown in Figure 6 is provided in Table S1. Detailed interpretation of more MS data is needed to better understand other significant implications, which are potentially present in our experimental results, of cancer biology and anticancer drug mechanisms; however, this goal is beyond the scope of current study.

CONCLUSION

We reported a study using combined microscale devices, the Micro-funnel and the Single-probe, to collect extracellular contents inside live spheroids for real-time MS analysis. The Micro-funnel is produced from laser-pulled fused silica capillary, a biocompatible material,

with small tip size ($\sim 25 \mu\text{m}$). This type of microscale device can be implanted into live spheroids cultured from cancer cells to collect the extracellular compounds in the inner region of spheroids. The spheroids were treated using the anticancer drug irinotecan under a series of conditions. The collected extracellular contents in the Micro-funnel were then efficiently extracted by the Single-probe, a microscale sampling and ionization device, for real-time MS analysis. We successfully detected irinotecan, drug metabolites, and cellular metabolites using our technologies. Statistical data analyses, including PCA and *t* test, have been carried out to obtain the overall features of cell metabolomic changes induced by anticancer drug treatment. Our results indicate that extracellular compounds in inner spheroid regions are significantly altered by irinotecan treatment and different treatment conditions result in significant differences in metabolic signatures. In particular, the increased abundances of phospholipids and glycerolipids reflect cellular responses to drug treatment and may indicate the drug resistance of central tumor cells. Our current study demonstrates that the combined Micro-funnel and Single-probe MS technique can be used to monitor extracellular metabolites in the microenvironment inside spheroids. However, we may not be able to completely eliminate the possibility of sampling intracellular molecules in experiments. Dead cells (e.g., due to the lack of oxygen and nutrients as well as the presence of drug molecules) may be present inside spheroids. Cellular contents released by dead cells can be mixed with extracellular metabolites and collected by the Micro-funnel in MS measurements. Future studies are necessary to test this possibility. This novel technique can potentially be applied to studies of live tissues in different areas, including fundamental research of the regulation of extracellular compounds in tumor development, drug penetration and metabolism in tumor treatment, and biomarker discovery in cancer diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Combined Micro-funnel and Single-probe technique for MS analysis of extracellular compounds in live spheroids. (a) A photo of a Single-probe device. (b) A photo of a Micro-funnel before and after being implanted into a spheroid. (c) Coupled Micro-funnel and Single-probe device. (d) Real-time MS analysis of extracellular compounds in a live spheroid using the coupled Micro-funnel and Single-probe device.

Figure 2. Detection of irinotecan and its metabolites from extracellular compounds accumulated by the Micro-funnel.

Figure 3.
Scheme of irinotecan metabolism in tumor cells. All metabolites were detected as the singly protonated species in our experiments.

Figure 4. Concentration- and time-dependent measurements of extracellular irinotecan and its metabolites in live spheroids. (a) The normalized ion intensities of irinotecan and its metabolites measured from spheroids treated using 5, 20.6, 50, 100, 200, 300, 400, and 500 μM irinotecan for 5 h. (b) The normalized intensities of irinotecan and its metabolites measured from spheroids treated for 5, 12, 24, and 48 h using the IC_{50} of irinotecan (20.6 μM). Results are the averaged values (with standard deviations) from eight replicates ($n = 8$) for each concentration and time length.

Figure 5.

Principle component analysis (PCA) of mass spectra. (a) PCA results of culture medium (fresh medium, medium after spheroids culture (control), and medium after drug treatment ($50 \mu\text{M}$ irinotecan)) and extracellular compounds in Micro-funnels (spheroids from control and drug treated ($50 \mu\text{M}$ irinotecan) groups). (b) PCA results of extracellular compounds in spheroids in concentrations-dependent experiments (20.6 , 300 , and $500 \mu\text{M}$). (c) PCA results of extracellular compounds in spheroids in time-dependent experiments (5 , 12 , and 48 h). Results are reported from eight replicates ($n = 8$) in each group, and each symbol represents an independent experiment.

Figure 6.

Heat maps summarizing the categorized extracellular lipids measured from spheroids under different treatment conditions. The compositions of extracellular lipids were significantly changed by anticancer drug treatment under (a) different concentrations (20.6, 300, and 500 μM) and (b) different treatment times (5, 12, and 48 h). Results are reported from eight replicates ($n = 8$) in each group. PA (phosphatic acid), PE (phosphoethanolamine), PG (phosphatidylglycerol), PS (phosphatidylserine), PI (phosphatidylinositol), PC (phosphatidylcholine), MG (monoglycerides), DG (diglycerides), TG (triglyceride), and SM (sphingomyelin).