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Letter

Mass Spectrometry Quantification of Anticancer Drug Uptake in Single Multicellular Tumor Spheroids

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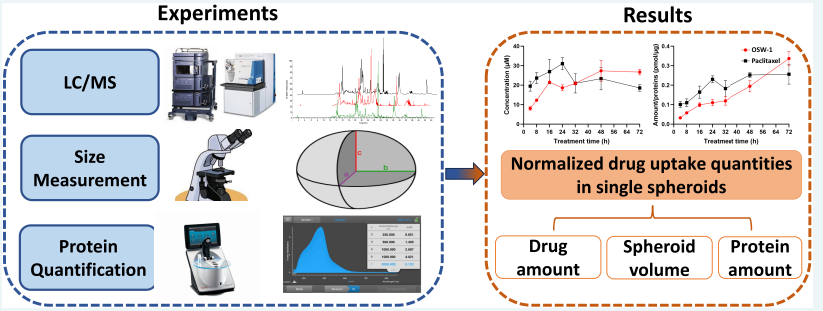

ABSTRACT: Although most advanced-stage ovarian cancers initially respond to platinum- and taxane-based chemotherapy, the majority of them will recur and eventually develop chemoresistance. Among all drug resistance mechanisms, reduced drug uptake in tumors is regarded as an important pathway acquired by drug-resistant cancer cells. For patients with ovarian cancer, chemoresistant cells can develop into multicellular spheroids and spread through ascite fluid that accumulates in their abdomen. These spheroids consist of 3D structures that are highly heterogeneous with different shapes, sizes, and compositions of cell types. Thus, studying drug uptake at the single spheroid level is important for understanding chemosensitivity and chemoresistance; however, drug-uptake studies in single spheroids have not been previously reported due to the lack of a suitable analytical technique. In this study, we cultured spheroids using the ovarian cancer cell line (OVCAR-8) and treated them using paclitaxel or OSW-1, a natural compound with anticancer properties. We then developed a method of quantifying drug uptake in single spheroids using LC/MS measurements and then normalized the drug amount in each spheroid to its size and total protein content. Our method can be used in translational studies of drug development, treatment, and prediction of drug efficacy prior to chemotherapy.

KEYWORDS: *single spheroids, drug uptake quantitation, ovarian cancer, LC/MS quantitation*

Despite significant advances in therapeutic options, ovarian cancer remains the most lethal of all gynecologic malignancies with an estimated more than 19,000 new cases and >13,000 deaths in 2023.[1] This is ascribed to the late stage at disease diagnosis due to the absence of effective screening methods, the complex, heterogeneous nature of the disease present at time of diagnosis and increasing with temporal evolution.[2,3] Most patients with ovarian cancer initially demonstrate exquisite chemosensitivity to standard of care frontline platinum- and taxane-based therapies with approximately 80% of patients experiencing a remission.[1,4] However, approximately 75% of those women experience a recurrence with only approximately 50% of patients alive 5 years following diagnosis.[1,4] Recurrences that occur more than 6 months following initial treatment typically respond to retreatment with chemotherapy; however, the cancers often recur with decreasing time intervals between each recurrence.[5] Patients with chemotherapy-resistant disease (recurrence <6 months from the last chemotherapy) have more limited options and a survival estimate of <17 months.[6,7]

The hallmark of advanced ovarian cancer is the accumulation of ascite fluid in the peritoneal space of the abdomen. Ovarian cancer cells can detach from the primary tumor and form multicellular spheroids (hereafter referred to as spheroids) in ascites,[8] which then spread and establish metastatic lesions in other pelvic organs.[9] In addition, spheroids often have higher levels of drug resistance, making treatment for ovarian cancer very challenging.[10] Although the exact mechanisms of the elevated drug resistance of spheroids are unclear, many aspects can influence it, such as mass density

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of the spheroid, extracellular matrix protein expression, and the drug uptake capacity in spheroids.[10] Because spheroids in ascites collected from ovarian cancer patients are heterogeneous with different sizes, mass densities, shapes, and compositions,[11] drug uptake measurements in single spheroids are inevitably needed. However, to the best of our knowledge, relevant studies have not been previously reported.

Chemoresistance of ovarian cancer spheroids is likely influenced by multiple factors, including the low proliferative state of cells inside spheroids,[12] overexpression of certain proteins promoting cancer cell survival (e.g., transglutaminase 2 prevents apoptosis induced by cisplatin),[13] and upregulation of multidrug resistant (MDR) pumps (e.g., ATP-binding cassette (ABC) transporters and P-glycoprotein).[14] It is critical to develop more effective chemotherapy drugs to overcome certain chemoresistance mechanisms, such as reduced drug uptake by spheroids, in the current ovarian cancer treatment. Recent studies performed by Bensen et al. showed that, unlike the standard-of-care reagent paclitaxel, the novel anticancer natural compound OSW-1 is more potent against an ovarian cancer cell line grown as a three-dimensional (3D) spheroid in comparison to cells grown as monolayers attached to cell culture plates.[15,16] It is not known, however, whether this increased potency is due to differential uptake of the drugs into cancer cells.

To elucidate all potential mechanisms of chemoresistance in ovarian cancer, quantifying drug uptake in single spheroids that are present among heterogeneous spheroids is needed. Our ultimate goal of this work is to develop suitable techniques to efficiently quantify drug uptake in single spheroids collected from patients with ovarian cancer. As a first step in this process, we used spheroids generated in culture dishes from a human ovarian cancer cell line to establish experimental methods prior to evaluating ascite specimens from patients with ovarian cancer. Spheroids obtained from 3D culture are regarded as a valid model system, compared with traditional 2D culture cell monolayers, to better mimic cell−cell interactions and the microenvironment in *in vivo* tumors.[17−19]

Evaluating drug uptake is one of the most important criteria for dose selection and dosing intervals in clinical applications.[20] Quantifying drug uptake can greatly promote our understanding of the pharmacodynamics and pharmacokinetics of cancer therapeutic drugs. A variety of different types of analytical techniques have been established for drug uptake measurement. Using fluorescence-labeled drug molecules,[21,22] liquid chromatography (LC),[23,24] gas chromatography (GC),[25] and capillary electrophoresis (CE)[26] systems coupled with different detectors, such as mass spectrometry (MS)[27,28] and UV detectors, the uptake of an anticancer drug can be determined.[29] Due to its incomparable sensitivity and accuracy in molecular analysis, MS allows for quantification of drug uptake in tiny amounts of samples, such as single cells.[16,30−32] However, only one study of drug uptake in single spheroids, which used Matrix Assisted Laser Desorption/Ionization (MALDI)-MS to quantify irinotecan,[33] has been reported. LC-MS has been proven as a powerful tool to quantify small molecules, such as drug compounds, drug metabolites, and cell metabolites, in biological samples such as tissues, cells, and biofluids.[34,35] Because spheroids, including *in vivo* samples and *in vitro* models, are heterogeneous with different sizes, mass densities, shapes, and compositions,[11] drug uptake measurements in single spheroids is inevitably needed. The data collected from a population of spheroids in a patient sample could then be used to evaluate how the profile of ascite spheroid shapes and sizes and their specific drug uptake properties are associated with patient treatment outcomes.

To achieve the goal of quantifying drug uptake in single spheroids, we developed an LC/MS-based method to quantify anticancer drug uptake in single spheroids. Spheroids were cultured using an ovarian cancer cell line and then treated with a novel natural product compound (OSW-1) and a traditional anticancer drug (paclitaxel). We used the LC/MS technique to acquire the absolute drug quantities in single spheroids spiked with isotopically labeled internal standard (IS). To compensate for the influence of spheroid size on drug uptake, we measured the volume of each spheroid and normalized drug uptake in each spheroid (i.e., intraspheroidal drug concentrations). However, the mass density of spheroids may vary; using their volume for normalization may not be sufficient to normalize data for comparisons among spheroids. Because the total protein amount is regarded as the robust reference to compare cell numbers,[36,37] we also quantified total proteins in each spheroid for drug uptake normalization. We observed different trends of uptake between these two drug compounds, which can be potentially used to understand their different efficacies when treating 3D spheroids.

The workflow of anticancer compound uptake quantification in single spheroids is illustrated in Figure 1. OVCAR-8 cells

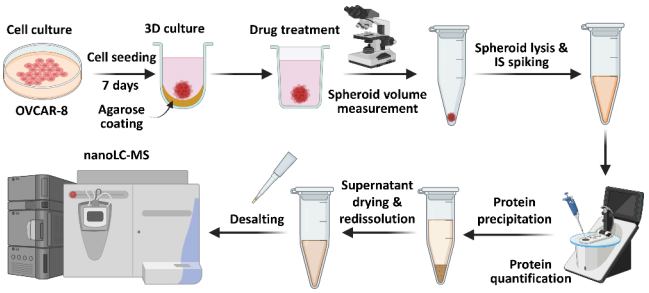

Figure 1. Workflow of anticancer drug uptake measurement in single spheroids (Created in BioRender. Yang, Z. (2024) BioRender.com/r69q185).

were used for 3D culture to produce spheroids, which were treated by anticancer compounds OSW-1 or paclitaxel. After the measurement of spheroids' volumes using a microscope, spheroids were lysed. A deuterated drug analogue (i.e., IS) was added into lysate at a known amount, followed by protein quantification, protein precipitation, drug extraction, and a desalting step. Quantification of drug compounds in lysates was performed by using a nanoLC/MS system.

Two anticancer drugs (paclitaxel and OSW-1 at 1.0 μM (IC_{50} at 72 h)) were used to treat spheroids at seven different treatment times (4, 8, 16, 32, 48, and 72 h), with five biological replicates for each treatment time. In total, 70 spheroids were analyzed. It is expected that the quantity of drug uptake in single spheroids is very low due to their small sizes; therefore, a nano LC/MS method, which provides better sensitivity and a lower limit of detection compared with regular LC/MS,[38] was used in the current study. Another challenge in these experiments was to accurately measure the volume of each spheroid. In previous studies, the volume of spheroids cultured using UW228−3 cells was calculated based on the assumption that all spheroids were spherical,[39] of which the volume can be readily calculated. However, this assumption may not be true

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for all spheroids cultured using other cell lines, and especially not in the heterogeneous nature of clinical ascites specimens. For example, spheroids cultured using the OVCAR-3 cell line possess irregular shapes, whereas OVCAR-8 cells show a more spherical shape, but it is still not ideal.[15] Thus, it is necessary to find a suitable way to accurately measure the volume of spheroids, allowing for the better quantification of drug uptake in single spheroids. In addition, we noticed that drug treatment affected the mass density of spheroids, resulting in changes in their volumes and inaccuracy in intraspheroidal drug concentrations, as detailed in the next section. To accurately correlate cell quantity and drug uptake, we used the total protein amount to normalize the drug amounts in each spheroid and then performed the comparison among all samples treated under different conditions.

SPHEROID VOLUME MEASUREMENT

Spheroids cultured in the current work possess ellipsoid chapes, of which lengths of the three semiaxes (i.e., *a*, *b*, and *c*) are different. We measured these three values for each spheroid using a calibrated inverted microscope and then calculated their volumes (Supporting Information, eq 1). Based on microscope images, we observed that the major axis (*a*) of all spheroids was ∼800 μm before the treatment, but it was either increased or decreased (ranging between 600 and 900 μm) after treatment. The minor axes (*b* and *c*) were ∼50−80% of their major axes (Figure S1) in all cases. In addition, the edge of the spheroids became coarse for the longer treatment time, likely due to the detached cells during drug treatment (Figure S2).

We noticed that paclitaxel and OSW-1 had similar influences on the spheroid volume (Figure 2). In most cases, changes of

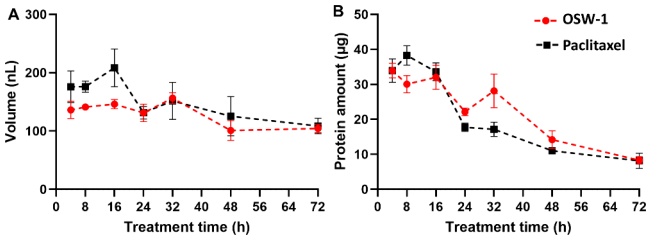

Figure 2. Influence of anticancer drug treatment on (A) spheroid volume and (B) total proteins in spheroids. 1.0 μM OSW-1 or paclitaxel was used for spheroid treatment. Five replicates were measured under each treatment condition.

spheroid volumes were not significant ($p > 0.05$) between two sequential time points during treatment (Figure S5). However, it is worth noting that anticancer drug treatment reduced cell density in spheroids (Figure S2), so using cell volume to normalize drug uptake in individual spheroids may result in bias. Although cell numbers in each spheroid could be a better way to normalize drug uptake amounts, it is challenging to accurately count live cells in each spheroid and then collect them for subsequent experiments. Instead, cell numbers in each spheroid can be correlated to the amounts of total proteins,[37] which is likely to be a more reliable way to normalize drug uptake in single spheroids.

PROTEIN QUANTITIES IN SINGLE SPHEROIDS

The total protein amounts in single spheroids were measured using a NanoDrop One. Our results show that each spheroid typically contained ∼30 μg of protein prior to drug treatment (Figure 2B), whereas the total protein amount generally decreased as the treatment time increased. Because the amount of proteins in the same type of cells is relatively stable, a decrease of the total protein amount indicates a loss of cell numbers in spheroids (Figure 2B). Both compounds caused a more rapid initial decrease in total protein per spheroid followed by a more gradual decrease after 24 h. In contrast, the effect of both compounds on the volume was more steady and gradual (Figure 2A).

DRUG CONCENTRATION

To compare the uptake of both drug compounds in spheroids with different sizes, we normalized the drug uptake amount to the volume of each spheroid and obtained an intraspheroidal drug concentration. Our results indicate that the intraspheroidal OSW-1 concentrations increased in the first 16 h and then steadily rose until 48 h. However, paclitaxel uptake in single spheroids reached its maximum at 24 h and then decreased in the subsequent treatment time (Figure 3A).

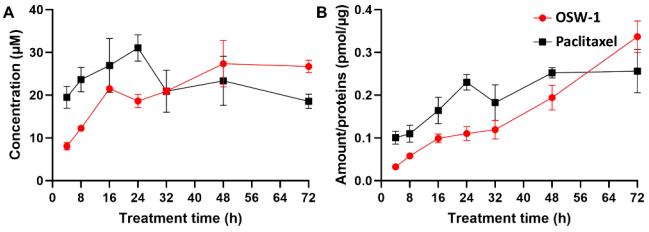

Figure 3. Drug uptake amounts in single spheroids normalized to (A) spheroid volume and (B) total proteins in the spheroid. 1.0 μM OSW-1 or paclitaxel was used for the spheroid treatment. Five replicates were measured under each treatment condition.

As shown in Figure 3A, intraspheroidal drug concentrations (8.0−27.6 μM for OSW-1 and 18.6−31.1 μM for paclitaxel) are significantly higher than their treatment concentrations (1.0 μM for both drug compounds), indicating that both drugs accumulated in cells during the treatment. However, trends of drug uptake affected by treatment time were clearly different between these two drugs. For OSW-1 (Figure 3A), the intraspheroidal concentration reached ∼25 μM at 16 h, remained at this level before 32 h, and then slightly increased upon longer treatment time. However, for paclitaxel, concentrations reached the highest level at 24 h and then kept decreased (Figure 3A). Although these comparisons provide valuable information on drug uptake, a direct comparison these trends and values could be biased because spheroid densities were affected by these two drugs at different levels.

DRUG AMOUNT NORMALIZATION TO TOTAL PROTEIN AMOUNT

For a comparison of drug uptake in spheroids unbiased by spheroid densities, the measured anticancer agent amount in each spheroid was normalized to its total protein amount (Figure 3B). Smaller analytical variations (Table S1) among the five spheroids (biological replicates) in each group were observed. Interestingly, even though the protein amount was reduced by the drug treatment suggestive of cell death, the amount of drug continued to increase. We observed distinct trends of drug uptake when normalized to protein amount

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between these two drugs. In the first 32 h, the normalized amounts of OSW-1 consistently increased throughout all treatment times, whereas the uptake of paclitaxel increased in the first 24 h but then reached a plateau regardless of longer treatment time. This 24-h time point inflection for plateauing of protein-normalized paclitaxel uptake matches the inverse inflection observed for the plateau of paclitaxel reduction of total spheroid protein amount. Different trends between these two drugs can likely explain why, compared with paclitaxel, OSW-1 is more effective at inhibiting OVCAR-8 spheroid growth, as reported in our previous studies.[15]

CONCLUSIONS

This study provided quantitative analyses of anticancer compound uptake in single ovarian cancer spheroids. Spheroids were cultured using an ovarian cancer cell line (OVCAR-8) and then treated by a front-line anticancer drug (paclitaxel) and a novel natural compound (OSW-1). We measured the absolute amounts of drug compounds in single spheroids and used either the spheroid volume or total protein amount in individual spheroids to normalize drug quantifications. We observed that these two drugs exhibited different trends of drug uptake. At the 24 h treatment time point, the effects of paclitaxel on decreasing spheroid volumes and protein amounts, along with drug uptake, plateau. In contrast, OSW-1 exhibited continuous gradual decreases in spheroid volumes and protein amounts, and nevertheless, the compound uptake continued to gradually increase. This observation may provide an explanation of why OSW-1 is more potent than paclitaxel in the treatment of spheroids cultured using multiple ovarian cancer cell lines, as shown in our previous study.[15] Our established methods have a high potential to predict drug efficacy by evaluating single spheroid drug uptake in ascite specimens. Spheroids, including those with irregular shapes, can be collected from ovarian cancer patients for *ex vivo* treatment of a series of different drug compounds, followed by drug uptake measurements. Within a few days, the measured drug uptake in spheroids can be used to predict which drugs are better options prior to actual lengthy chemotherapy. Furthermore, information gained about associations between spheroid sizes and shapes with drug uptake, along with evaluation of the heterogeneous spheroid population in individual patients with cancerous ascites, could be utilized to optimize drug choices and dosing in an effort to maximize drug uptake in the spheroid population, which should translate into improved anticancer efficacy. It is worth noting that the isotopically labeled compounds were used as ISs for reliable MS quantification in the current work. However, if this type of IS is not readily available, other quantification approaches (e.g., using molecules with similar structures as the IS or standard addition method) can be adopted with carefully constructed calibration curves.[30]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.4c00525.

- Experimental details of spheroid culture, drug treatment, volume measurement, quantification of total proteins, drug extraction, and LC/MS analysis.
- Photos of spheroids, workflow of protein quantification, LC/MS detection of OSW-1 and its internal standard, and influence of drug treatment on spheroids' volume (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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