Quantitative Size-Based Analysis of Tumor Spheroids and Responses to Therapeutics

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ABSTRACT

Drug resistance remains a major clinical problem despite advances in targeted therapies. In recent years, methods to culture cancer cells in three-dimensional (3D) environments to better mimic native tumors have gained increasing popularity. Nevertheless, unlike traditional two-dimensional (2D) cell cultures, analysis of 3D cultures is not straightforward. Most biochemical assays developed for 2D cultures have to be optimized for use with 3D cultures. We addressed this important problem by presenting a simple method of quantitative size-based analysis of growth and drug responses of 3D cultures of cancer cells as tumor spheroids. We used an aqueous two-phase system to form consistently sized tumor spheroids of colorectal cancer cells. Using spheroid images, we computed the size of spheroids over time and demonstrated that growth of spheroids from this analysis strongly correlates with that using a PrestoBlue biochemical assay optimized for 3D cultures. Next, we cyclically treated the tumor spheroids with a MEK inhibitor, trametinib, for 6-day periods with a recovery phase in between. This inhibitor was selected because of mutation of colon cancer cells in the MEK/ERK pathway. We used size measurements to evaluate the efficacy of trametinib and predict development of resistance of colon cancer cells during the cyclical treatment and recovery regimen. This size-based analysis closely matched the biochemical analysis of drug responses of spheroids. We performed molecular analysis and showed that resistance to trametinib emerged due to feedback activation of the PI3K/AKT signaling pathway. Therefore, we combined trametinib with a PI3K/AKT inhibitor, dactolisib, and demonstrated that size-based analysis of spheroids reliably allowed quantifying the effect of the combination treatment to prevent drug resistance. This study established that size measurements of spheroids can be used as a straightforward method for quantitative studies of drug responses of tumor spheroids and identifying drug combinations that block resistance.

Keywords: aqueous two-phase system, tumor spheroid, size-based analysis, drug resistance, combination treatment

INTRODUCTION

Cancer cells cultured as a monolayer offer a simple *in vitro* model to screen chemical compounds during drug discovery. However, cancer cells cultured in a two-dimensional (2D) environment differ structurally, biologically, and functionally from those residing in complex three-dimensional (3D) tumors.^{1–3} This disparity is a major cause of high attrition rates of compounds in the process of drug discovery.³ To overcome this technological limitation, there has been a significant interest in developing 3D cultures that better mimic native tumors. Culturing cancer cells as tumor spheroids is one of the most widely used methods. Spheroids closely recapitulate solid tumors in terms of close cell–cell contacts, cell–matrix interactions, spatial gradients of oxygen and nutrients due to diffusion limitations resulting in both proliferative and dormant cells, hypoxia, and drug penetration limitations.^{3–5}

Although spheroids are now widely used in drug testing studies, incorporating them in mainstream drug discovery has been challenging due to the difficulty of mass production of homogeneously sized spheroids, convenient maintenance of spheroids for long-term studies, and straightforward biochemical analysis of drug responses of cells.³ Biochemical analysis of spheroids primarily relies on colorimetric, luminescence, and fluorescence assays that were originally developed for monolayer cell cultures.⁶ Their use with spheroid cultures requires assay optimization to ensure sufficient penetration of assay reagents into spheroids. Considering inherent differences in cell–cell adhesions of different cancer cells and resulting differences in the formation and compactness of their spheroids, these assays should be optimized with spheroids of different cell lines to obtain an optimum dynamic range. Another challenge with some of these biochemical assays is inconsistencies that result from multiple addition, aspiration, and wash steps. There is still a need for simple and straightforward techniques that enable analysis of growth and drug responses of tumor spheroids.³ To overcome these challenges, we propose the use of a size-based analysis of spheroids to predict drug responses of cells in spheroids.

We have recently developed a spheroid culture technology using an aqueous two-phase system (ATPS) consisting of aqueous solutions of polyethylene glycol (PEG) and dextran (DEX).^{7–9} A nanodrop of the DEX phase containing cancer cells is dispensed into the immersion PEG phase. Due to its higher density, the aqueous DEX phase settles at the bottom of the nonadherent culture vessel and forms a stable and round drop.⁷ Ultralow interfacial tension between the two aqueous solutions effectively maintains cancer cells in the DEX phase nanodrop and leads to spontaneous formation of a fully viable spheroid.¹⁰ We adapted this technology to robotic liquid handling to facilitate mass production of spheroids in microwell plates.¹¹

In this study, we used this technology to form highly consistent spheroids of HT-29 colorectal cancer cells and show that analysis of 2D images of spheroids can be used to reliably predict the growth of spheroids. Furthermore, we treated spheroids with several molecular inhibitors of deregulated kinase pathways in the cells and demonstrated that spheroid size-based quantification of drug effects is consistent with biochemical analysis of drug-treated spheroids. Analysis of the size of spheroids from 2D images strongly correlated with the fluorescent signal resulting from metabolic reduction of a PrestoBlue reagent by cells, when spheroids maintained a compact shape with defined boundaries. As a novel application of this approach, we showed that the size-based quantification method reliably predicts adaptive resistance of colorectal tumor spheroids during cyclical treatments with kinase inhibitors, and the effectiveness of combination drug treatments to overcome drug resistance. With increasing interest in targeted therapies of cancers,^{12–15} this simple approach will benefit studies that aim to elucidate concentration ranges of small-molecule inhibitors that produce toxic effects and accordingly select effective concentrations for downstream molecular studies. Overall, our study suggests that size-based quantitative analysis of spheroids in cancer research and drug discovery will simplify workflow and help identify effective drug compounds.

MATERIALS AND METHODS

Cell Culture

HT-29 and HCT116 colorectal cancer cells (ATCC) were cultured in McCoy's 5A medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), 1% streptomycin/penicillin (Thermo Fisher Scientific), and 1% glutamine (Thermo Fisher Scientific). Cells were cultured in a humidified incubator at 37°C with 5% CO₂ and subcultured when they were 80%–90% confluent. A 0.25% trypsin solution (Thermo Fisher Scientific)

was used to dissociate cells from culture flasks. The complete growth medium was used to neutralize trypsin. The resulting cell suspension was centrifuged down at 1,000 rpm for 5 min at 4°C. After removing the supernatant, cells were suspended in 1 mL of the culture medium and counted using a hemocytometer before spheroid formation.

Spheroid Formation

Aqueous solutions of 5.0% (w/v) PEG (Mw: 35 kDa) and 6.4% (w/v) DEX (Mw: 500 kDa) were used to form spheroids in 384-well, ultralow attachment round-bottom plates (Corning) using our established ATPS technology, as described before.^{8,9} Polymer solutions were prepared with the complete growth medium. Formation of a single spheroid within each DEX drop depends on cell density and DEX drop volume.⁹ Therefore, 1.5×10^4 cells in 0.3-μL DEX drops were used to result in one spheroid per well.

Quantification of Metabolic Activity, Size, and Drug Resistance of Spheroids

To evaluate growth of HT-29 spheroids, a PrestoBlue reagent was added daily for 10 days to each well at 10% of total well volume. The plate was incubated for 4 h to allow cells to metabolize the reagent and produce a fluorescent compound.⁸ The fluorescent signal was measured using a plate reader (Synergy H1M; Biotek Instruments) and averaged over 14 replicates each day. Additionally, the spheroids were imaged using an inverted fluorescence microscope (Axio Observer; Zeiss). Phase microscopy was used to image spheroids and measure their diameter from 2D images. The largest z-plane at which spheroids had a sharp boundary was selected for imaging. To quantify drug resistance, phase images of spheroids were captured at the end of each treatment cycle. The diameter of each spheroid was measured using ImageJ (NIH) and converted to volume assuming a spherical shape.¹⁶ To quantify the efficacy of each treatment cycle, the volume of treated spheroids was normalized with the volume of vehicle control (nontreated) spheroids and the result was expressed as a percent volume.

Biochemical and Morphological Measurements of Drug Responses

We selected trametinib, sorafenib, dactolisib, and ponatinib as kinase molecular inhibitors against HT-29 spheroids. All four compounds were purchased from Selleckchem. Stock solutions of inhibitors were prepared according to the manufacturer's instructions. All compounds were dissolved in dimethyl sulfoxide (DMSO) except for dactolisib, which was dissolved in dimethylformamide. The stock solutions of trametinib, dactolisib,

ponatinib, and sorafenib were 10, 10, 50, and 50 mM, respectively. Stock solutions were stored at -80°C . For dose-response experiments, 1, 10, 100 nM, 1, 10, and 50 μM concentrations of inhibitors were used. At the highest concentration of drugs used in our experiments, DMSO concentration did not exceed 0.5%. These inhibitors were tested dose-dependently against HT-29 spheroids according to our protocol previously described.¹⁶

Cyclical Treatment and Recovery of Spheroids

We cyclically treated HT-29 spheroids with 5 nM trametinib. The two rounds of cyclical treatments were designated as treatment 1 (T1) and treatment 2 (T2). The recovery period was designated as recovery 1 (R1). Each phase lasted 6 days. Each treatment phase included drug addition to spheroids at the beginning and a drug renewal 72 h later, whereas the recovery phase included culture medium addition to spheroids at the beginning and culture medium renewal 72 h later. Concentration of each drug was maintained constant during the treatment phases. For T1, spheroids were treated with trametinib at a 5 nM concentration. At the end of T1, the culture medium containing the inhibitor was thoroughly removed from microwells and replaced with fresh medium. At the end of R1, spheroids were treated with 5 nM trametinib again. In parallel, spheroids from R1 received fresh medium only to serve as a vehicle control for T2. The volume of trametinib-treated spheroids was normalized to that of nontreated spheroids and expressed as a percent volume.

Combination Treatments of Spheroids

Trametinib was used in combination with dactolisib. The IC_{50} value for each inhibitor against HT-29 spheroids was obtained from its respective dose-response curve. Trametinib and dactolisib were combined at fixed concentration ratios of multiples (0.25, 0.5, 1, 2, 4) of their respective IC_{50} values. Solutions with these concentrations were made by serially diluting stock solutions in the culture medium. Each combination of concentrations for a pair of drugs used 14 replicates. In parallel to each combination treatment, spheroids of HT-29 cells were also treated with trametinib or dactolisib alone. Next, images of spheroids were captured to estimate the volume of spheroids. Both vehicle control (nontreated) and drug-treated groups had 14 replicates. The volume of spheroids treated with each concentration of an inhibitor was normalized to that of nontreated spheroids and expressed as percent volume. GraphPad Prism 5 was

used to fit a four-parameter, sigmoidal dose-response curve to the percent volume data and to determine the area under the dose-response curve (AUC).¹⁷ A combination index (CI) was used to determine synergism between combinations of inhibitors.^{17,18}

Western Blot and Immunohistochemical Analysis of Spheroids

Western blot analysis of spheroids was performed according to our established protocol.¹⁶ Primary antibodies for phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2), phospho-AKT (Ser473), and AKT (pan) (C67E7) were purchased from Cell Signaling Technology. Solutions of primary antibodies were prepared at concentrations recommended by the manufacturer. Membranes were incubated overnight at 4°C with primary antibody solutions. After repeated washing, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h, followed by repeated washing. Detection was carried out using an ECL chemiluminescence detection kit (GE Health Care) and FluorChem E imaging system (ProteinSimple). Immunohistochemical analysis was performed as previously described.¹⁹

Statistical Analysis

Student's *t*-test between T1 and T2 treatments ($p < 0.05$ defining significance level) and Pearson's correlation coefficient between the volume of spheroids and the corresponding fluorescent signal from the PrestoBlue assay were performed using Microsoft Excel.

RESULTS AND DISCUSSION

Microprinting of Tumor Spheroids

The aqueous DEX phase nanodrop containing cancer cells remained immiscible from the PEG phase solution and facilitated aggregation of cancer cells into a compact spheroid within 48 h (Fig. 1A–C). The ATPS allows free diffusional influx of nutrients into the drop phase to nourish cells and efflux of waste products of cells into the immersion phase. The ATPS microprinting approach provides a mild environment for cells to form spheroids containing fully viable cells.^{8,20} Adding a drug solution or medium reduced concentrations of PEG and DEX polymers below a threshold concentration and resulted in a single aqueous phase containing the spheroid (Fig. 1D).⁹ Thus, this approach was solely used to quickly and conveniently micropattern spheroids. Medium exchange every 3 days provided fresh nutrients and removed waste products of cells.

Fig. 1. Microprinting of tumor spheroids with ATPS technology. **(A)** Aqueous DEX phase drop containing cancer cells is dispensed into a well containing an aqueous PEG phase. **(B)** Immiscibility of DEX and PEG solutions maintains cancer cells confined to the DEX phase drop to aggregate into a spheroid. **(C)** HT-29 colorectal cancer cells self-assemble to form a compact spheroid within the DEX phase drop. **(D)** Adding a drug solution or culture medium reduces concentrations of polymers and results in a single medium phase containing the spheroid. Scale bar is 250 μm (**C**, **D**). ATPS, aqueous two-phase system; DEX, dextran; PEG, polyethylene glycol.

Size Consistency and Growth of Tumor Spheroids from Size Measurements

Conventional spheroid-forming techniques such as liquid overlay and spinner flask do not produce spheroids of a consistent size. The inconsistent shape of spheroids can impact reproducibility of drug testing data.²¹ We adapted our technology to robotic liquid handling to conveniently form large quantities of consistently sized spheroids in 384-microwell plates.¹¹ With a density of 1.5×10^4 HT-29 cells per DEX drop, a variation of less than 5.5% from an average diameter resulted (*Fig. 2A*). HT-29 spheroids had distinct boundaries and a round and compact shape. With a similar starting cell density, our technology generated spheroids in flat-bottom well plates that were significantly more compact than spheroids generated in conventional ultralow attachment plates over a 10-day culture period (*Supplementary Fig. S1*). Our biochemical analysis using the PrestoBlue assay showed progressive increase in cellular metabolic activity of spheroids for a period of 10 days, indicating cell proliferation in spheroids. The measured signal increased by ~ 2.6 -fold during culture. Our morphological image analysis showed an increase in the average diameter of spheroids from 440 μm on day 1 to 729 μm on day 10. Assuming a spherical shape for spheroids, this corresponded to ~ 4.5 -fold increase in the volume of spheroids. The lower fold change in fluorescence signal intensity compared with the change in the volume of spheroids is likely due to the difficulty of penetration of PrestoBlue reagent into compact spheroids, which can lead to decrease in the fluorescence signal intensity. We obtained no significant fold change difference in the volume of spheroids and fluorescence signal intensity until day 8, at which the average size of spheroids was 664 μm . This is consistent with limited penetration of biochemical dyes into spheroids larger

than 650 μm in diameter.²¹ We further confirmed the validity of these results by immunostaining cryosections of HT-29 spheroids for proliferative cell marker Ki-67 (*Fig. 2C*). Importantly, the fluorescence intensity and volume data showed a linear correlation with a coefficient of $R^2 = 0.94$ (*Fig. 2B*). This suggests that size-based analysis of spheroids is an alternative metric to quantify cell proliferation.

A major advantage of size-based analysis of growth of spheroids is the use of significantly fewer spheroids compared with biochemical assays such as MTT, PrestoBlue, and CellTiter-Glo. Unlike biochemical assays that need a new set of spheroids at each analysis time point due to addition of biochemical reagents, the same spheroids can be imaged over time with the size-based analysis method. This analysis, which is consistent with few other studies using size of spheroids,^{22,23} will be very useful especially during long-term cultures with repeated treatments of spheroids. We note that the presence of a hypoxic or necrotic core can affect the precision of size-based analysis of growth of spheroids, but this effect will be minimal because the core zone constitutes a relatively small volume of a spheroid. For example, assuming that a spheroid of $\sim 500 \mu\text{m}$ diameter (such as that in *Fig. 2c*) has a hypoxic core of 200 μm diameter, the hypoxic core constitutes only $\sim 6\%$ of the volume of the spheroid. For large spheroids with a larger hypoxic or necrotic core, this error will increase. However, for the size of spheroids used in this study, the size-based analysis provides a precise estimate of growth of spheroids.²⁴

Predicting Treatment Outcomes Using Size of Spheroids

We treated HT-29 spheroids dose-dependently with specific kinase molecular inhibitors. The concentration of DMSO at the

Fig. 2. Size consistency and growth of spheroids. **(A)** Measured diameter of HT-29 spheroids in 384-well plates after 48 h of spheroid formation. Each bar represents the average diameter of spheroids from a column of a 384-well plate. Each error bar represents the standard deviation from the mean value. **(B)** Relationship between the metabolic activity of spheroids from a PrestoBlue biochemical assay and size of spheroids measured from 2D images over a 10-day culture. R^2 denotes the goodness-of-fit parameter. *Inset* images are representative spheroids from different days. **(C)** Ki-67 staining of HT-29 spheroids shows proliferative cells. Scale bar is $200 \mu\text{m}$ (**B**, **C**). 2D, two-dimensional.

highest concentration of molecular inhibitors used in the dose-dependent experiments did not exceed 0.5%. This DMSO concentration did not have a significant effect on viability of cells in spheroids after 6 days of culture (*Supplementary Fig. S2*). We selected these molecular inhibitors because HT-29 cells have mutations in several kinase pathways,²⁵ potentially making cells sensitive to protein kinase inhibitors. Biochemical analysis using PrestoBlue showed that molecular inhibitors reduced growth of spheroids to different extents and with different efficacies (*Fig. 3A, B*). Trametinib was the most effective inhibitor against HT-29 spheroids at low nanomolar concentrations of $<10 \text{ nM}$. Above this concentration, spheroids were either fluffy or disintegrated. Similarly, sor-

afenib was effective above 100 nM drug concentrations, whereas ponatinib and dactolisib showed efficacy at micromolar concentrations. To determine a correlation between these metabolic activity-based results and size of spheroids, we selected spheroids from drug concentrations that were effective, but did not disintegrate the spheroids. We found that the volume of spheroids treated with these inhibitors linearly correlated with the fluorescent signal from the PrestoBlue assay. The goodness-of-fit parameter (R^2) demonstrated a linear correlation (*Fig. 3C–F*). We found that Pearson's correlation coefficients between dose-dependent reduction in volume of spheroids and fluorescence intensity from the biochemical analysis were 0.975, 0.978, 0.966, and 0.986 for

Fig. 3. Size measurements of spheroids reliably predict outcomes of treatments with molecular inhibitors. **(A)** Dose–response of HT-29 spheroids based on a PrestoBlue biochemical assay. **(B)** The target of each inhibitor and the respective IC₅₀ value from its dose–response experiment with HT-29 spheroids are listed in the table. **(C–F)** Correlation between average values of the fluorescent signal from a PrestoBlue assay and volume of spheroids from morphological images ($n=14$). R^2 represents the goodness-of-fit parameter. Different data points in each graph represent different drug concentrations. Only those concentrations that did not disintegrate the spheroids were considered.

treatments with trametinib, sorafenib, ponatinib, and dactolisib, respectively. To demonstrate broad utility of the size-based analysis with other cell lines, we treated HCT116 colorectal cancer spheroids with trametinib. Again, $R^2=0.99$ demonstrated a strong linear correlation (*Supplementary Fig. S3*). We found that Pearson's correlation coefficient between dose-dependent reduction in volume of spheroids and fluorescence intensity from the biochemical analysis was 0.998 after treatment with trametinib. This analysis substantiates the validity of morphological analysis of drug-treated spheroids to predict treatment outcomes.

Evaluating Drug Resistance to Intermittent Treatments Using Size of Spheroids

Next, we validated the utility of size-based analysis of spheroids to predict treatment outcomes by evaluating drug resistance of tumor spheroids. Our dose-dependent tests identified trametinib (MEK1/2 inhibitor) as the most effective inhibitor against HT-29 spheroids (*Fig. 3A, B*). Thus, we used this inhibitor to model drug resistance of cancer cells. We used an intermittent dosing method and treated HT-29 spheroids with trametinib for two 6-day

periods separated by a recovery phase (*Fig. 4A*). We captured images of spheroids for both vehicle control and treated groups (*Fig. 4B*, inset images) and quantified the volume ratio of treated to vehicle control spheroids, that is, percent volume, during the two treatment rounds. We found that the 5 nM trametinib treatment reduced the volume of spheroids by ~77% during T1. However, the same drug concentration reduced the volume by ~71% during T2 (*Fig. 4B*). We further validated this finding using the biochemical PrestoBlue assay, which showed that the viability of spheroids during T2 was ~1.29-fold greater than that during T1 (*Supplementary Fig. S4*). This significant decrease in efficacy of trametinib ($p<0.05$) indicates that HT-29 cells in spheroid culture adapt to and become less sensitive to trametinib treatment. Interestingly, our drug resistance model reliably emulated several *in vivo* studies that have shown that cyclical treatment of tumor xenografts with molecular inhibitors of MEK1/2 did not reduce the tumor size, necessitating other treatment options.^{26,27}

Considering that single-agent treatment with trametinib becomes less effective during successive treatments,

Fig. 4. Size measurements of spheroids predict resistance of colorectal tumor spheroids to intermittent treatments. **(A)** The schematic shows the experimental protocol for two cycles of treatment of HT-29 spheroids with trametinib separated by a recovery phase. **(B)** A fixed concentration (5 nM) of the inhibitor was used for each of the two treatment rounds. Inset images above each bar in the graph show representative images of untreated (left) and treated spheroids (right). * Represents statistical significance measured at $p < 0.05$, $n = 14$. **(C)** Molecular analysis shows feedback signaling through the PI3K/AKT pathway in spheroids treated with 5 nM trametinib during treatment 1 (T1). **(D)** Molecular analysis shows feedback signaling through the PI3K/AKT pathway in spheroids treated with 5 nM trametinib during treatment 2 (T2). Spheroids from R1 were used as vehicle control during T2. Scale bar in **(B)** images is 200 μ m.

understanding the molecular basis of resistance of cells is critical to design effective treatments. Various studies have shown that MEK inhibition can reactivate the PI3K/AKT signaling pathway.^{28–30} This prompted us to analyze AKT protein activity in HT-29 spheroids cyclically treated with trametinib. As expected, trametinib treatment downregulated phosphorylation of ERK1/2, which is consistent with the decrease in size of HT-29 spheroids during T1 (Fig. 4C). However, unlike in vehicle control spheroids, the phosphorylated AKT level dramatically increased in trametinib-treated HT-29 spheroids (Fig. 4C). Trametinib treatment during T2 also led to persistent upregulation of pAKT (Fig. 4D). This established that targeting the MEK/ERK pathway using trametinib results in feedback activation of the PI3K/AKT pathway. Thus, HT-29 spheroids under intermittent treatment with trametinib rely on the PI3K/AKT pathway to proliferate and survive.

Analysis of Combination Treatment Using Size of Spheroids

Next, we evaluated the potential of a combination treatment to block drug resistance of HT-29 spheroids to single-agent therapy. We used trametinib and a PI3K/mTOR inhibitor, dactolisib, both in combination and in single-agent treatments. Using the IC_{50} value from each single-agent treatment (Fig. 3B), we treated spheroids with multiples of the IC_{50} concentration of each compound and measured the size of spheroids at the end of a 6-day treatment period. We constructed dose–response curves using spheroid size and computed the AUC from each treatment (Fig. 5A). Except for the $4 \times IC_{50}$ combination concentration that resulted in fluffy spheroids, images of spheroids at all other concentrations were used for the analysis (Fig. 5B). Compared with treatments with trametinib and dactolisib alone that gave AUC values of 0.346 and 0.350, respectively, the combination of inhibitors

Fig. 5. Size measurements of spheroids predict the efficacy of combination treatment of colorectal tumor spheroids. **(A)** Combination treatment of HT-29 spheroids with trametinib and dactolisib significantly decreased the size of spheroids compared with the respective single-agent treatments. **(B)** Images of spheroids after dose-dependent treatments with each inhibitor alone and their combination. **(C)** Synergy plot shows CI versus Fa. **(D)** Combination treatment of HT-29 spheroids with trametinib ($2 \times IC_{50}$) and dactolisib ($2 \times IC_{50}$) downregulated ERK and AKT activities. Scale bar in **(B)** images is 300 μ m. CI, combination index; Fa, fraction affected.

significantly enhanced the response by $\sim 19\%$ (AUC value 0.160). This was also evident from morphological images of spheroids.

To elucidate whether reduction in the size of spheroids in the combination treatment was synergistically regulated by the two compounds, we computed a combinations index (CI): CI < 1 indicates synergism, CI = 1 shows an additive effect, and CI > 1 represents antagonism between a pair of compounds.¹⁸ Figure 5C shows that the decrease in volume of spheroids by the trametinib/dactolisib pair is synergistic at all combinations of concentrations used. We next performed Western blot analysis at the $2 \times IC_{50}$ combination concentration, at which we observed the highest reduction in the size of spheroids. Our molecular analysis showed downregulation of phosphorylated ERK1/2 and AKT by $\sim 68\%$ and $\sim 40\%$, respectively, using the combined trametinib and dactolisib treatment for 24 h (Fig. 5D). Therefore, targeting of these signaling pathways by combining specific MEK and PI3K inhibitors synergistically reduced the size of HT-29 spheroids and

feedback signaling of MEK/ERK and PI3K/AKT pathways. Our result indicates the importance of simultaneous blocking of compensatory signaling pathways to block drug resistance to single-agent therapies. Importantly, our result from morphological measurements is consistent with several animal model studies,^{26,31} establishing that size measurements of spheroids can be used as a convenient method to reliably predict the efficacy of different drug treatment modalities.

The size-based analysis of drug-treated spheroids can become significantly beneficial when performing high-throughput, long-term combination treatments with molecular inhibitors to identify effective pairs. Such experiments require using each compound at multiple doses, each with a sufficient number of replicates to enable reliable statistical analyses. An experiment with each pair of compounds often requires several hundred spheroids multiplied by the number of measurement time points if biochemical assays are used.^{30,32} Screening different pairs of inhibitors will need an

exceedingly large number of spheroids. On the other hand, using the size-based method will significantly reduce the quantity of samples and labor associated with production and maintenance of cultures in such long-term experiments. Availability of high-content imaging systems will speed up imaging and quantification of the size of spheroids.²⁴

CONCLUSION

Our spheroid printing technology resulted in consistently sized spheroids in a high-throughput format. Quantitative analysis of the size of spheroids closely correlated with the metabolic activity of spheroids and reliably emulated spheroid growth. Importantly, the size-based analysis predicted treatment outcomes of drug tests when spheroids had a round shape with a defined boundary. In a proof-of-concept study to model cyclical drug treatment and recovery of tumor spheroids, our size-based analysis of spheroids demonstrated that single-agent treatment with a targeted kinase inhibitor leads to adaptive drug resistance of cancer cells. After identifying the molecular mechanism of drug resistance, we rationally designed a combination treatment to block feedback signaling induced by single-agent drug treatment and significantly reduce the size of spheroids. We note that the precision of this analysis method depends on a round morphology of spheroids. Therefore, it will be specifically useful for drugs that shrink or prevent growth of spheroids and do not disintegrate them. Incorporating this straightforward size-based analysis of spheroids in drug treatment studies will significantly reduce the use of biochemical assays with spheroid cultures and streamline identification of effective drugs and combinations of drugs that prevent the growth of cancer cells.

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DISCLOSURE STATEMENT

No competing financial interests exist.

SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4

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Abbreviations Used

2D = two-dimensional
 3D = three-dimensional
 ATPS = aqueous two-phase system
 AUC = area under the dose-response curve
 CI = combination index
 DEX = dextran
 DMSO = dimethyl sulfoxide
 ERK = extracellular signal-regulated kinase
 Fa = fraction affected
 MEK = mitogen-activated protein kinase
 PEG = polyethylene glycol