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Development, validation and pilot screening of an in vitro multi-cellular three-dimensional cancer spheroid assay for anti-cancer drug testing

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ABSTRACT

It has been demonstrated that two-dimensional (2D) monolayer cancer cell proliferation assay for anti-cancer drug screening is a very artificial model and cannot represent the characteristics of three-dimensional (3D) solid tumors. The multi-cellular in vitro 3D tumor spheroid model is of intermediate complexity, and can provide a bridge to the gap between the complex in vivo tumors and simple in vitro monolayer cell cultures. In this study, a simple and cost-effective cancer 3D spheroid assay suitable for small molecule anti-cancer compound screening was developed, standardized and validated on H292 non-small lung cancer cell line. A pilot screening with this assay was performed utilizing a compound library consisting of 41 anti-cancer agents. The traditional 2D monolayer cell proliferation assay was also performed with the same cell line and compounds. A correlational study based on the IC_{50} values from the 2D and 3D assays was conducted. There is low correlation with the two sets of biological data, suggesting the two screening methods provide different information regarding the potency of the tested drug candidates.

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1. Introduction

Cell based assays are widely used in anti-cancer drug screening processes. These assays significantly reduce animal usage and costs in drug development.[1] Most often, screening assays to determine the potency of anti-cancer drugs are performed on cells growing on two-dimensional (2D) glass or plastic platforms. This highly artificial cellular environment cannot accurately mimic three-dimensional (3D) in vivo tumor environment, in which cancer cells reside.[2] Therefore, the traditional 2D cell culture is believed to have limited predictive value for anti-cancer drug screening. Because cancer cells lose part of their phenotypic and functional characteristics when grown in 2D monolayer culture, the artificial 2D cell proliferation assays may provide inaccurate data regarding the anti-cancer potency of chemotherapeutic agents.[3] This observation dramatically affects the susceptibility of the cells when exposed to anti-cancer drugs. In addition, drugs can enter the cells in 2D monolayer culture relatively easier, than in 3D tissue-like cell clusters. All these factors affect drug potency. The limitations of 2D cell proliferation assays promote strong interest of researchers to develop and evaluate the more complex in vitro 3D cellular assay, which can better reflect the human tumor tissue environment and provide better accuracy for anti-cancer drug testing.[3–5] In recent years, the recognition of the potential of 3D cell assays to be incorporated into the mainstream of developmental processes for new anti-cancer drugs has increased. If well standardized and pre-tested, 3D cellular assays could become widely adopted into anti-cancer drug discovery. This will significantly improve pre-animal and pre-clinical drug selection and provide more reliable prediction of the most promising drug candidates.[4]

In vitro multi-cellular 3D spheroid tumor models have been proven to be more physiologically relevant to in vivo tumors.[3,4] Firstly, tumor cells in 3D spheroids have much stronger cell-cell interaction and adopt a different morphology compared to the cells in 2D monolayer culture.[6] More specifically, cancer cells in 2D culture exhibit an unnatural spread morphology, while cancer cells in 3D culture show a clustered, spheroid morphology that is similar to in vivo tumors.[7] Secondly, the growth rate of tumor cells in 3D culture better reflects in vivo tumors, as opposed to the growth rate of tumor cells in 2D culture. The nutrients and oxygen needs to penetrate the multi-cellular layers in 3D spheroids to support cell growth, a feature that mimics the in vivo delivery system. In contrast, 2D cultured cells are exposed to excess nutrition and oxygen and proliferate faster than in vivo tumors. Thirdly, cancer cells cultured in the 3D model show differences in anti-cancer drug sensitivities compared to cells in 2D culture.[5,8] The drugs have to penetrate the multi-layers of cells to reach the inner part of the 3D spheroids, which partially mimics the in vivo drug delivery systems.[9] The strong cell-cell interactions in 3D also improve the

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survival rates of the cancer cells when treated with anti-cancer agents;[10] which contributes to the relatively low drug sensitivity of the 3D spheroid model. The anti-cancer drug activity in 3D culture closely mimics the way in vivo tumors respond to chemotherapeutic treatment. Apparently, the multi-cellular 3D spheroid culture has multiple advantages over 2D cell culture when selecting the most promising anti-cancer drug candidates from a large pool of compounds. Therefore, multi-cellular 3D spheroid culture screening may even replace some animal tests.

There are several methods including liquid overlaying, hanging drops, and microencapsulation techniques to culture cells on non-adherent surfaces in order to form multi-cellular 3D spheroids.[4,5,7,11,12] Many spheroids generated from these techniques, however, suffer from problems such as low efficiency spheroids formation, limited culturing duration and extreme variations in spheroid sizes.[5,7,11,12] In addition, many developed in vitro multi-cellular 3D spheroid assays were not validated with a compound library to examine their effectiveness.[5] So far, 3D spheroid assays have not yet been incorporated into mainstream drug development processes due to the complex methodological requirements and unconfirmed reliability.

The main aim of the present study is to provide a simple and standardized in vitro multi-cellular 3D spheroid tumor assay suitable for small molecule drug candidate screening. An assay was successfully developed with a non-small cell lung cancer cell line H292, selected from seven cancer cell lines. To validate this model, the 3D spheroids were treated with a group of well-known tubulin inhibitors that showed significant in vivo tumor growth suppression activity in other studies.[13–15] The effects of these chemotherapeutic agents were evaluated on spheroid growth. Consequently, a tubulin inhibitor library (41 compounds) developed in our laboratory was tested on 3D spheroid models to validate the effectiveness of this assay.[16,17] The conventional 2D monolayer cell proliferation assay was also performed with the same cell line and compounds. The correlation of the IC_{50} values from the 2D and 3D assays were analyzed. Several compounds showed significant inhibition activity to 3D spheroid growth.

2. Results and discussions

2.1. H292 non-small cell lung cancer cells formed very well packed multi-cellular 3D spheroid

To develop an easy handling 3D spheroid assay, the ability of multiple cancer cell lines including lung cancer cell line A549 and H292, breast cancer cell line MCF-7 and MDA-MB-231, colon cancer cell line HT29, prostate cancer cell line PC-3 and trampC1; to form spheroids was evaluated. Most of these cell lines have been reported to easily form spheroids with liquid overlay technique, an easy performing experiment.[5,7,18] Although morphologically 3D spheroids seemed to form with these cell lines, the spheroids were not well packed. Some of them could be easily broken into pieces only by slight pipetting. In fact, these so-called spheroids were just cell-aggregated clusters, rather than well-organized tissue-like tumors. Fortunately, H292 cells showed the best spheroid morphology in shape and were also well packed (Fig. 1); which made them resistant even to very intense pipetting.

To check the correlation between H292 lung cancer 3D spheroid size and corresponding cell number, in vitro tumors were formed with various numbers of cells and the spheroid volumes were examined. The cells were liquid overlaid in 96 well plates covered with 1.5% agarose gel for 72 h. Spheroid images were taken and the volumes were calculated based on their diameters (Fig. 1). The spheroid volumes were proportional to the cell numbers. With approximately 20,000 cells, the spheroid diameter was about 200 μm and the interwell variation in spheroid diameter was under 5%. The variation in the mean spheroid diameter of three independent experiments was below 10%. When the cell number reached 30,000 or above, the formed spheroids showed ellipsoid or other irregular shapes (images not shown). It was difficult to estimate these types of spheroid volume based on their images.

2.2. Growth kinetics of H292 3D spheroids

To be a useful 3D spheroid model, the growth kinetics of the in vitro tumor is very critical. Spheroids growing too slowly will create difficulties when conducting anti-cancer drug screenings with the model.[5] The growth kinetics of the H292 spheroids formed with various cell numbers were examined. To our surprise, the spheroids did not grow at all, even when they were incubated with 20% FBS for more than 10 days. The morphology of the spheroids also did not change; and they still remained as well packed spherical shapes, resistant to very intense pipetting. It has been reported that extracellular matrix (ECM, Matrigel) contributes to the growth of spheroids formed with MDA-MB-231 breast cancer cells.[7,18] Without Matrigel, MDA-MB-231 cells cannot form spheroids and grow.[18] For H292 cells, 1.5% agarose gel coating of the plate successfully induced spheroid formation, but did not accelerate spheroid growth. Apparently, H292 cells have a much stronger ability to form spheroids compared to other cell types. To stimulate spheroid growth, Matrigel was added to the culture medium of the H292 spheroids at various concentrations and identified the concentration of 2.5% (weight/volume) as the best one. The in vitro tumor exhibited very nice growth kinetics under the Matrigel formulated medium (Fig. 2). The high cell number (20,000 cells) spheroids showed a very rapid growth pattern and reached a diameter of about 800 μm, starting at 200 μm in 12 days. The low cell number spheroids (4000 cells) also grew very well, but in a much slower manner. It was also noticed that the interwell variation in spheroid diameter was much larger for the spheroids formed with high cell numbers after 12 days of growth. To develop the best assay for the following studies, 10,000 cell spheroids was chosen as the ideal model, which showed acceptable growth kinetics and limited interwell variation. Therefore, 10,000 cells, 2.5% Matrigel formulated culture medium were the best combination for the spheroid condition. The cell viability of the spheroids was also checked with MTS assay, which has been demonstrated as an effective method in another study.[19] Surprisingly, the H292 lung cancer spheroids did not show strong ability to convert the MTS reagent. It may be due to the well-packed tissues that limit the dye to penetrate the spheroids, which also limits the release of the converted dye into the medium. Therefore, in the following studies, 3D spheroid assay was standardized based on the changes in spheroid size.

2.3. Validation of H292 3D spheroids with well-known anti-cancer agents (tubulin inhibitors)

To validate the effectiveness of the H292 3D spheroid as a model for anti-cancer drug screening, tests were performed with several well-known tubulin inhibitors, since these types of agents are commonly used in clinics for the treatment of non-small cell lung cancer.[20] Paclitaxel, Indibulin, ABT751, and Colchicine that showed significant in vivo anti-cancer activity,[13–15,21,22] were tested with both monolayer cell proliferation assay and also the 3D spheroid growth assay. As shown in Figure 3A, all four tubulin interfering agents significantly inhibited H292 monolayer cell proliferation and 3D spheroid growth. Based on other studies, chemotherapeutic agents always show better potency in 2D monolayer cell proliferation assays.[5,19] However, it seems that the tubulin inhibitors were not significantly more potent in the 2D assay than in the 3D assay in the current study. It was possibly due to the time of the treatment. In the 2D proliferation assay, the treatment lasted

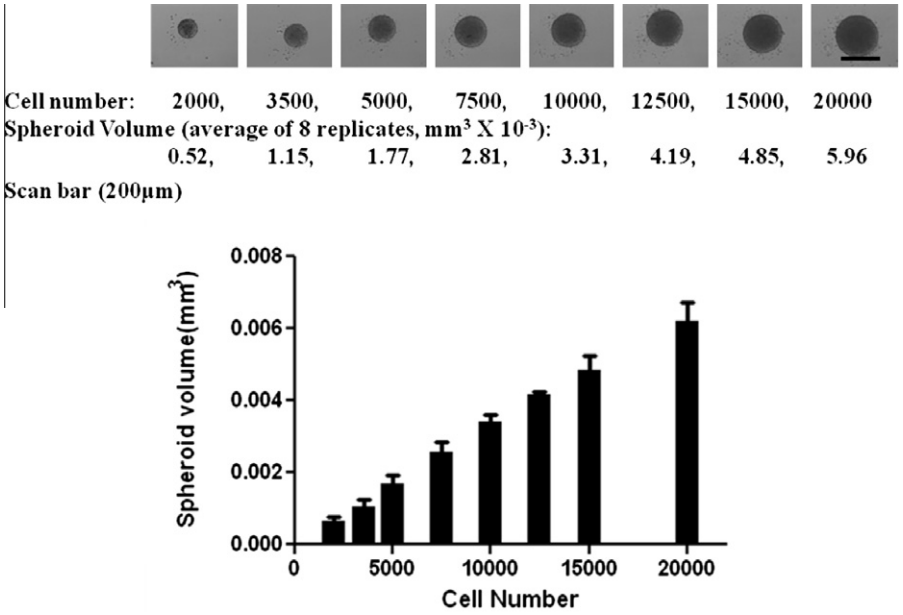


Figure 1. Morphology of the multi-cellular 3D spheroid and the correlation between cell number and spheroid volumes. The images were taken after the spheroid formed at day 3. The cell number showed a tight proportionality to the spheroid volume. Magnification: 10 X objective, scan bar: 200 μm.

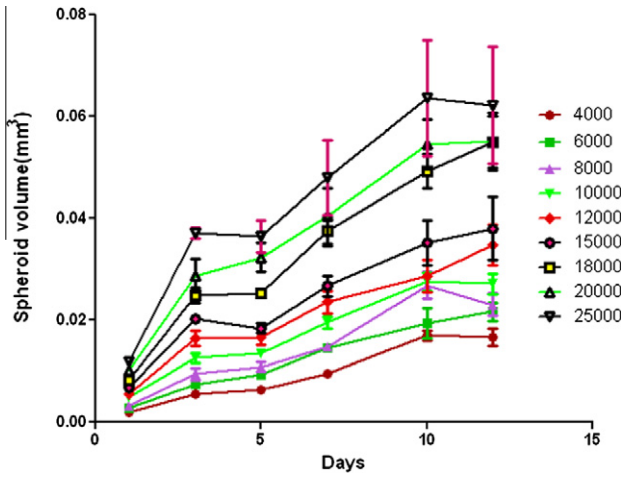


Figure 2. Growth kinetics of spheroids with different cell number. The cells were overlaid in 96-well flat-bottom plates coated with 70 μL of a 1.5% agarose (weight/volume) solution in distilled water. After the spheroids were formed in 48 h, they were then treated with growth factor–reduced Matrigel™ formulated culture medium, resulting in a final volume of 200 μL with 2.5% Matrigel. Spheroid morphological images were taken every other day. Data points are means ± SD for three individual experiments with four replications. The volume of the spheroid was calculated based on the diameters.

only 3 days versus 7 days of treatment for the 3D spheroid growth assay. Nevertheless, these agents dose-dependently inhibited the 3D spheroid growth. More importantly, the integrity of the spheroids were not affected by the treatments (Fig. 3B). Even for ABT751 at 50 μM and Paclitaxel at 100 nM, the spheroids maintained their spherical shapes at the end of the treatment (other agents gave similar results, images not shown). The spheroid integrity is very critical for the evaluation of the outcome of the treatment, despite spheroid size measurement or other methods used such as MTS and Acid Phosphatase Assays to normalize the results.[5,19,23] Furthermore, the spheroids expressed smooth growth patterns with different concentrations of the compound treatments (Fig. 3C, the growth curve of Paclitaxel and ABT751 treated spheroids were listed as representatives). The easy handling H292 in vitro 3D multi-cellular spheroid assay has the potential to be used as a model for chemotherapeutic agent screening.

2.4. Anti-cancer agents pilot screening with H292 3D spheroids

It is very important to pilot test the assay with a compound library before it can be widely used for anti-cancer drug screening. A sulfonamide tubulin inhibitor library with 41 compounds have been developed previously.[16,17] These compounds have similar structures and inhibit tubulin polymerization. A detailed structure and anti-cancer activity relationship (SAR) of these compounds had been summarized based on the monolayer cell proliferation assay with breast cancer cells.[16,17] To test if the newly generated in vitro 3D spheroid assay could be suitable for high throughput screening, a pilot screening with the tubulin inhibitor library was conducted. To further elucidate whether the 3D spheroid model is more advanced than the 2D monolayer cell proliferation assay for anti-cancer drug discovery, 2D cell proliferation assay with the same compounds and cell line was performed for comparison purposes. The IC_{50} values generated from the two assays are listed in Table 1. The potency difference index (IC_{50} inhibiting 2D cancer cell proliferation/IC_{50} inhibiting 3D spheroid growth) is summarized in Table 1 as well. Generally, chemotherapeutic agents show better potency in 2D cell proliferation assay than in 3D spheroid growth assay.[5,19] However, most of the anti-cancer agents tested in this study did not show better potency in the 2D cell proliferation assay. It was speculated that the compounds have decreased potency in the 3D spheroid assay as well. The relatively longer time of exposure of spheroids to the compounds contributes to the lower IC_{50} values. To determine the correlation of the two studies, the two sets of data, that is, IC_{50}s from 2D proliferation assay and IC_{50}s from 3D spheroid assay were analyzed. As illustrated in Figure 4, there is no strong correlation between the 2D and 3D data; with the correlation coefficient of 0.353. The results indicate that the 3D spheroid assay provides a different potency trend of the compounds compared to the 2D monolayer screening. The 3D data should be more reliable to determine the more promising drug candidates. However, it is very difficult to summarize a SAR for the 3D spheroid assay results based on the IC_{50} values generated. Multiple factors affect the potency of the compounds in the 3D

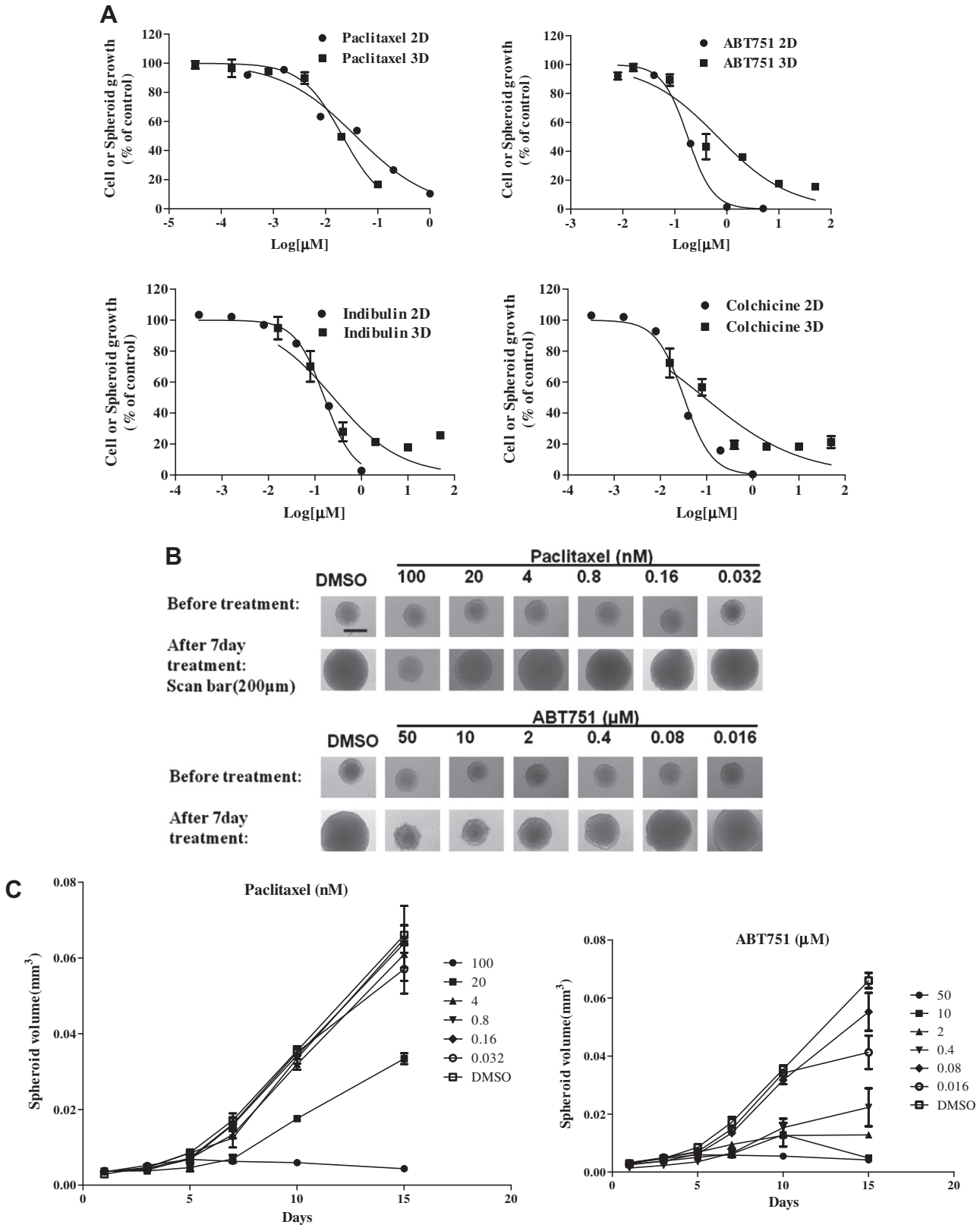


Figure 3. The effects of anti-cancer drugs in 3D spheroid culture. (A) Cell viability in H292 monolayer cells after 3-day treatment and spheroid volume in H292 3D culture after 7-day treatment with four tubulin interfering agents including Paclitaxel, Indibulin, ABT751, and Colchicine. Data points are means ± SD for three individual experiments with four replications. IC_{50} values were calculated to emphasize the differences in drug potency in 2D verses 3D cultures. Paclitaxel, IC_{50} of 37.86 ± 8.26 nM for 2D and 21.17 ± 3.75 nM for 3D; Indibulin, IC_{50} of 159.6 ± 13.9 nM for 2D and 249.3 ± 124.4 nM for 3D; ABT751, IC_{50} of 177.2 ± 17.3 nM for 2D and 670.2 ± 257.6 nM for 3D; Colchicine, IC_{50} of 32.60 ± 4.45 nM for 2D and 90.62 ± 48.67 nM for 3D. (B) 3D spheroid integrity following treatment with Paclitaxel and ABT751. Representative phase contrast imagines of H292 multi-cellular 3D spheroid at the onset of the treatment and after a 7-day treatment interval with various concentrations. Magnification: 10 X objective, scan bar: 200 μm. (C) Dose and time dependent curves of Paclitaxel and ABT751 with 3D spheroid assay.

Table 1
Suppression of monolayer cancer cell proliferation and multi-cellular spheroid growth with sulfonamide tubulin inhibitors

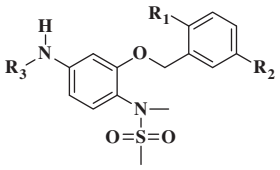
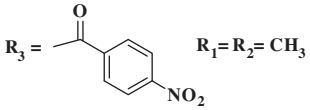
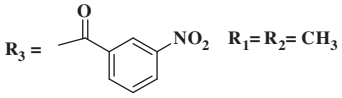
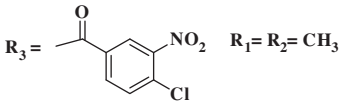
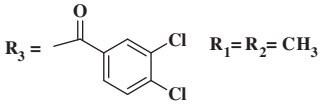
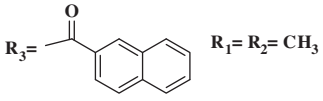
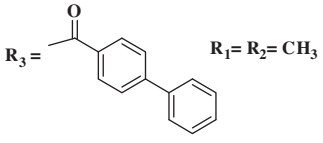
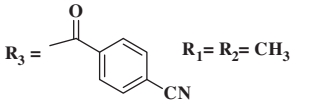
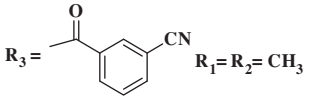
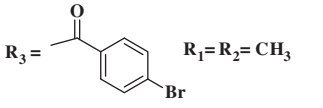
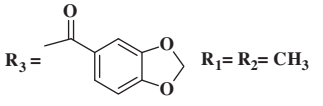
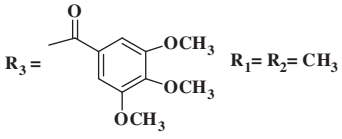
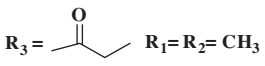
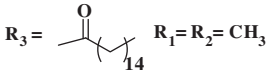
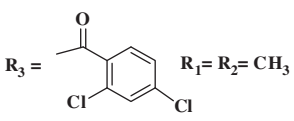
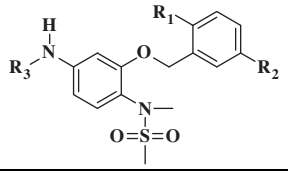
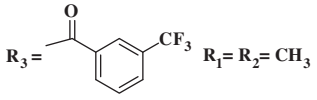
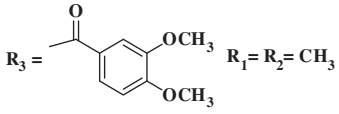
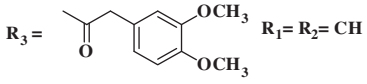
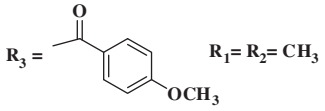
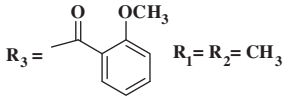
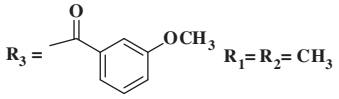
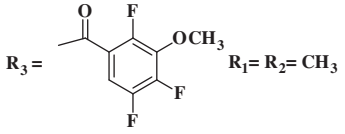
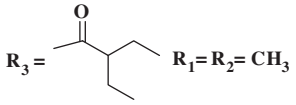
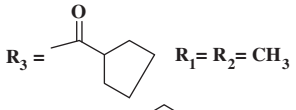
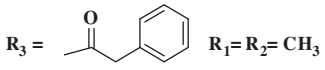
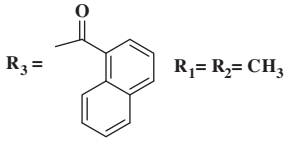
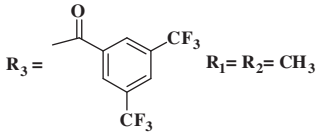
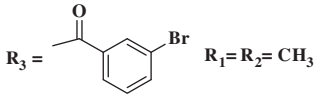
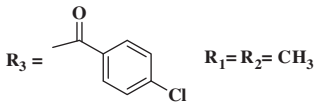
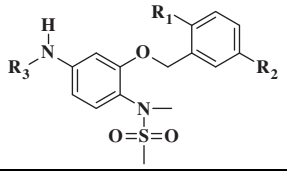
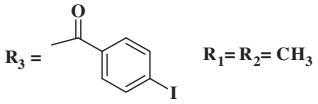
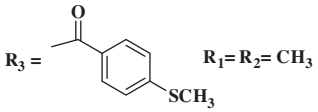
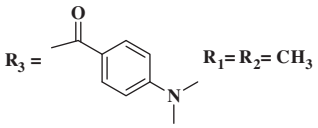
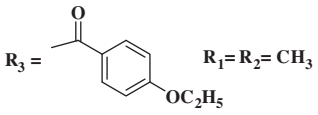
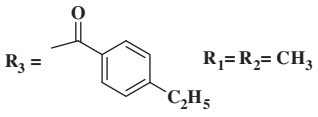
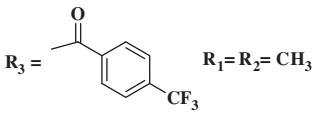
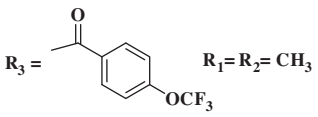
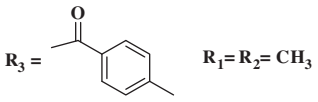
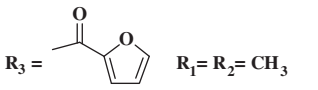
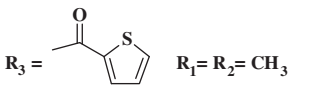
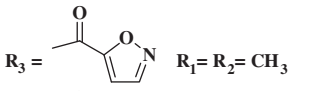
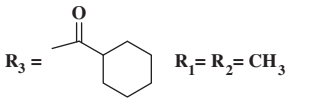
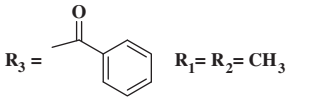
Entry		IC_{50} against 2D monolayer cell proliferation (μM) 3 days	IC_{50} against 3D spheroid growth(μM) 7 days	IC_{50} of monolayer cells/IC_{50} of spheroid
1		14.9 ± 6.49	19.61 ± 9.76	0.76
2		9.82 ± 3.89	2.07 ± 0.58	4.74
3		7.51 ± 5.98	26.8 ± 14.6	0.28
4		4.48 ± 1.81	0.39 ± 0.22	11.5
5		0.38 ± 0.18	0.24 ± 0.09	1.58
6		4.12 ± 1.41	6.41 ± 4.99	0.64
7		5.75 ± 1.95	7.60 ± 3.20	0.75
8		11.04 ± 3.55	3.38 ± 1.23	3.27
9		1.24 ± 0.08	0.69 ± 0.24	1.79
10		0.39 ± 0.13	0.77 ± 0.27	1.70
11		1.21 ± 0.42	0.71 ± 0.35	1.70
12		52.73 ± 20.5	>200	
13		36.9 ± 5.85	>200	
14		13.30 ± 7.63	2.52 ± 1.63	5.23

Table 1 (*continued*)

Entry		IC_{50} against 2D monolayer cell proliferation (μM) 3 days	IC_{50} against 3D spheroid growth(μM) 7 days	IC_{50} of monolayer cells/IC_{50} of spheroid
15		14.44 ± 5.58	7.65 ± 3.9	1.89
16		0.64 ± 0.17	0.20 ± 0.05	3.2
17		91.5 ± 27.1	>200	
18		0.57 ± 0.32	0.17 ± 0.05	3.35
19		14.45 ± 7.80	1.04 ± 0.36	13.9
20		20.67 ± 7.50	2.44 ± 1.52	8.47
21		5.21 ± 2.12	4.54 ± 3.28	1.14
22		58.2 ± 9.44	>200	
23		14.8 ± 2.27	25.1 ± 8.64	0.59
24		44.7 ± 12.9	>200	
25		1.85 ± 0.81	1.63 ± 0.44	1.13
26		1.85 ± 0.81	3.37 ± 1.75	2.28
27		16.5 ± 2.38	70.24 ± 54.8	0.23
28		1.29 ± 0.43	0.99 ± 0.33	1.30

(*continued on next page*)

Table 1 (*continued*)

Entry		IC_{50} against 2D monolayer cell proliferation (μM) 3 days	IC_{50} against 3D spheroid growth(μM) 7 days	IC_{50} of monolayer cells/IC_{50} of spheroid
29		0.06 ± 0.03	0.13 ± 0.07	0.46
30		0.13 ± 0.04	0.13 ± 0.06	1.00
31		0.90 ± 0.23	0.59 ± 0.27	1.53
32		0.21 ± 0.09	0.23 ± 1.36	0.91
33		1.58 ± 0.68	6.25 ± 2.93	0.25
34		0.71 ± 0.25	5.14 ± 3.66	0.14
35		0.18 ± 0.06	1.67 ± 1.02	0.11
36		2.63 ± 0.71	2.70 ± 1.55	0.97
37		13.03 ± 3.07	10.13 ± 5.71	1.29
38		23.3 ± 4.17	43.5 ± 31.7	0.54
39		23.8 ± 3.22	38.5 ± 28.9	0.62
40		7.99 ± 3.64	22.1 ± 11.0	0.36
41		14.7 ± 3.75	8.37 ± 2.66	1.76

H292 non-small lung cancer cells were used in the two assays. For monolayer assay, the treatment lasted 3 days; for 3D spheroid assays, the treatment lasted 7 days. The treatment was quart replicated and repeated three times. IC_{50} ± SD were performed using nonlinear regression analysis.

spheroid assay. For example, hydrophobicity has less effect on the 2D monolayer assay, but it dramatically affects the delivery of the compounds into the inner layer of the spheroids.[9] The toxicity of the compounds in 3D assay, in this case, cannot show a good trend correlated to the structure. Nevertheless, the in vitro 3D multicellular spheroid assay with a compound library including 41 anti-cancer agents was successfully validated. The compounds dose-dependently suppressed the growth of the spheroids, and

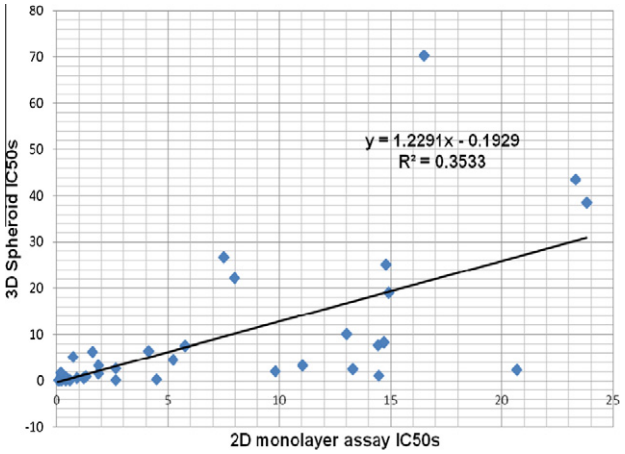


Figure 4. Correlation study of inhibitory effects of sulfonamide tubulin inhibitors on 2D monolayer cell proliferation and 3D spheroid growth.

provided very important parameters to select the best drug candidates. However, the image taking method to normalize the outcome of the treatment is difficult to be standardized in an automatically manner, which may limit the high throughput screening potential of the assay. In addition, the assay is only suitable for the screening of chemotherapeutic agents. It is not very useful for the development of targeted therapy, since the growth pathways in the spheroids are not elucidated yet.

2.5. Comparison of spheroid growth inhibitory activities of several potent lead compounds to identify the best drug candidates

The anti-cancer potency of the compounds was examined with the 3D spheroid assay, and several leads were also identified to be promising drug candidates to enter the in vivo xenograft testing. IC_{50} values of 0.2 μM were used as a cutoff and compounds with IC_{50} values around or below 0.2 μM were selected to perform the dose and time-dependent 3D spheroid growth assay lasting for a

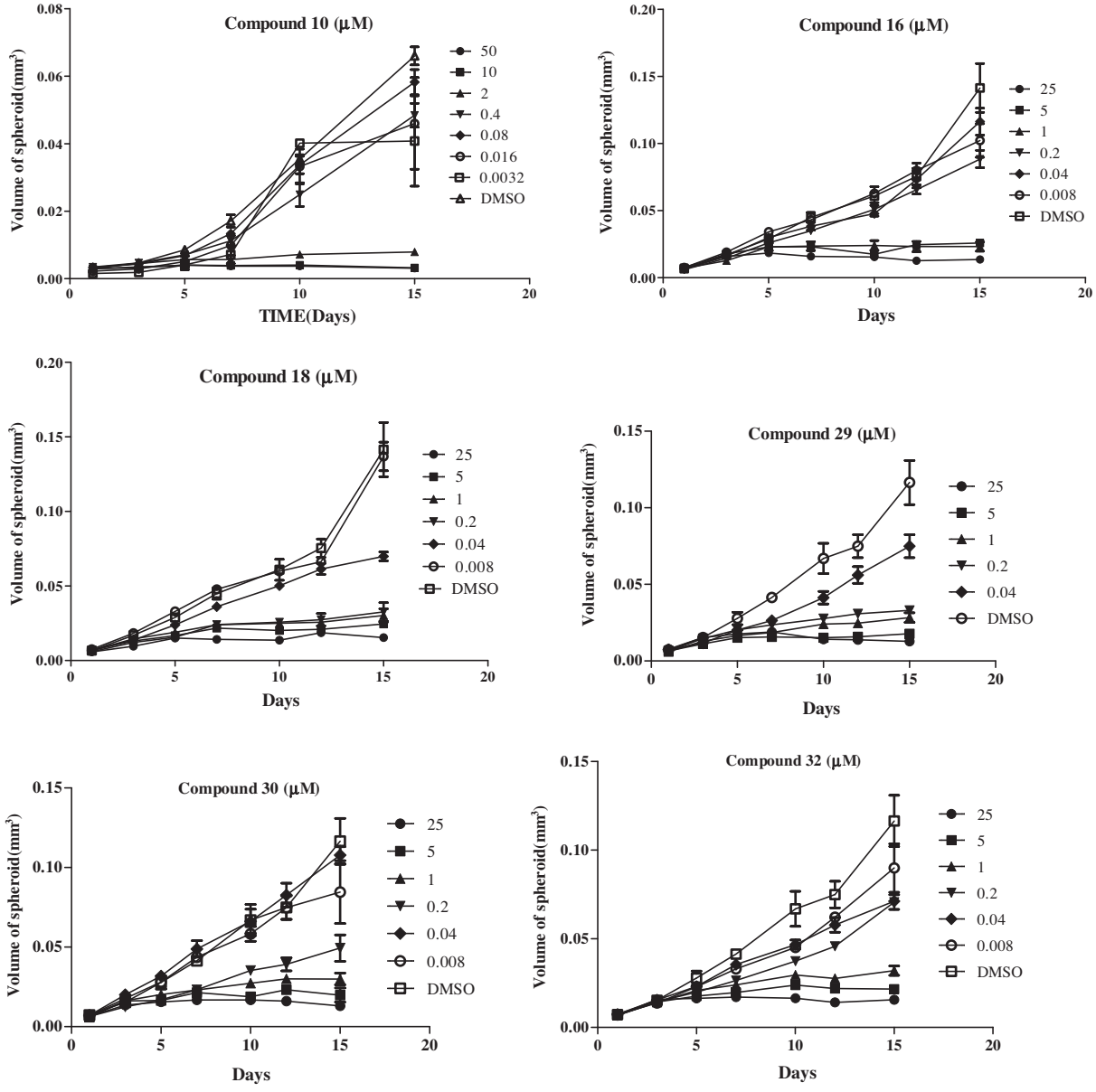


Figure 5. Dose and time dependent inhibitory effects of several lead compounds. Compound 10, which has been previously tested with in vivo assay, is included as a positive control. Data points are means ± SD for 3 individual experiments with four replications. The volume of the spheroid was calculated based on the diameters.

period of 15 days. Within the 41 agents, only compounds **16**, **18**, **29**, **30** and **32** were in this category. The time and dose-dependent inhibitory effects of these compounds on 3D spheroid growth were exhibited in Figure 5. Compound **10**, which has been tested with in vivo xenograft with 5 mg/kg dosage,[16] was also included as a positive control. It suppressed more than 90% of the spheroid growth at 2 μM. Compound **16** inhibited about 90% spheroid growth at 1 μM, but it was less active at 0.2 μM with only 35% suppression of the growth, so it may have a better in vivo potency than compound **10**. Compounds **18** and **29** showed the best activity, suppressed spheroid growth about 90% at 0.2 μM which is about 10-folds more active than compound **10**. Compounds **30** and **32** inhibited spheroid growth about 50% at 0.2 μM, which is less active than compound **18** and **29**, but apparently they are more potent than compound **10**. Therefore, compounds **18** and **29** are better drug candidates and have the potential to show potent in vivo anti-tumor activity.

3. Conclusion

A 3D multi-cellular spheroid assay with H292 lung cancer cells mimicking the aspects of in vivo–like tumor growth was developed and standardized. The assay was optimized to form uniform single spheroids in 96-well microplate with very minor interwell variation, suitable for high throughput screening. In addition, the formed spheroids were very well packed and sustained through very intense pipetting. The spheroids in the assay exhibited smooth growth curves under the treatment of different well-known chemotherapeutic agents. No disintegrity of the spheroid morphology was observed, even after long-term treatment. Furthermore, this assay was validated through pilot screening using a 41 compound library of sulfonamide tubulin inhibitors. A correlation study was performed based on the data generated from the treatment of 2D monolayer cell proliferation and 3D spheroid growth. A very poor correlation coefficient constant was generated, suggesting the 3D spheroid assay provided different potency information than 2D assay. The results support the importance of the incorporation of 3D cell culture in the drug discovery pipeline as a model to identify the best drug candidates. This in vitro multicellular 3D spheroid assay has the potential to be widely adopted in the drug discovery research due to its easy handling, and better prediction characteristics.

4. Materials and methods

4.1. Reagents

Tubulin inhibitors including Paclitaxel, Indibulin, ABT751, and Colchicine were purchased from Selleck (Houston, TX). The sulfonamide tubulin inhibitors were synthesized in our lab previously.[16] Matrigel Matrix was purchased from BD Biosciences (San Jose, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was from Sigma-Aldrich (Milwaukee, WI). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was from Promega (Madison, WI).

4.2. Cell culture

H292 lung cancer cells were obtained from ATCC (Rockville, MD). The cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 U/mL penicillin–streptomycin. FBS was heat inactivated for 30 min in a 56 °C water bath before use. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

4.3. Cell viability analysis

The effects of the tubulin inhibitors on H292 cell proliferation in the 2D monolayer culture were assessed using MTT assay in four replicates. 3000 cells per well were seeded in RPMI1640 medium in 96-well, flat-bottomed plates for 24 h, and were exposed to various concentrations of tubulin inhibitors dissolved in DMSO (final concentration $\leqslant$0.1%) in medium for 72 h. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μl of 0.5 mg/ml of MTT reagent in fresh medium, and cells were incubated in the CO_2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced MTT dye was solubilized in 200 μl/well DMSO. Absorbance was determined at 570 nm on a plate reader. For the 3D spheroid assay, the MTS reagents (Promega) was added (20 μL per well) after the 7 day treatment was over, and 100 μL supernatant of the medium from each well was transferred to a new 96 well plate. Absorbance was determined at 490 nm on a plate reader.

4.4. 3D culture spheroid formation and corresponding drug treatment

Uniform single-spheroid H292 lung carcinoma cells were cultured as follows. The 96-well flat-bottom plates were coated with 70 μL of a 1.5% agarose (weight/volume) solution in distilled water (freshly autoclaved). During the coating process, the agarose solution was maintained at $\geqslant$60 °C followed by cooling and setting at room temperature for 40 min. Then the cells were plated at a density of 2000–20,000 cells/well in 80 μL of RPMI-1640 (10% FBS), and allowed to form spheroid in 48 h. The spheroids were then treated with 20 μL of a 25% solution of growth factor–reduced Matrigel™ in cell culture medium,[18] resulting in a final volume of 100 μL with 5% Matrigel. Spheroids were cultured for one more day to reach an average diameter of 100 μm under standard tissue culture conditions (37 °C, 5% CO_2). For spheroid kinetics studies, 100 μL fresh medium was added at day 3 (final matrigel concentration became 2.5% at this stage); for drug treatment, 100 μL fresh medium with various concentration of drugs were added at day 3 (final matrigel concentration became 2.5% as well). Either Paclitaxel or ABT751 was used as a positive control on each assay. Spheroid morphological images in 96-well microplate were carried out manually on an inverted VWR VistaVision microscope (Bridgeport NJ) equipped with VWR VistaVision camera DV-2D. Spheroid diameters and volumes were determined from their images. The treatment was quart replicated, and the spheroid images were taken every other day. The suppression of the spheroid growth was normalized with control treatment (0.1% DMSO).

4.5. Statistical analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). IC_{50} values were determined using nonlinear regression analysis.

Acknowledgments

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