TITLE:

Characterization of the Effects of Migrastatic Inhibitors on 3D Tumor Spheroid Invasion by High-Resolution Confocal Microscopy

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SUMMARY:

The effects of migrastatic inhibitors on glioma cancer cell migration in three-dimensional (3D) invasion assays using a histone deacetylase (HDAC) inhibitor are characterized by high-resolution confocal microscopy.

ABSTRACT:

Drug discovery and development in cancer research is increasingly being based on drug screens in a 3D format. Novel inhibitors targeting the migratory and invasive potential of cancer cells, and consequently the metastatic spread of disease, are being discovered and considered as complementary treatments in highly invasive cancers such as gliomas. Thus, generating data enabling the detailed analyses of cells in a 3D environment following the addition of a drug is required. The methodology described here, combining spheroid invasion assays with high-resolution image capture and data analysis by confocal laser scanning microscopy (CLSM), enabled detailed characterization of the effects of the potential anti-migratory inhibitor MI-192 on glioma cells. Spheroids were generated from cell lines for invasion assays in low adherent 96-well plates and then prepared for CLSM analysis. The described workflow was preferred over other commonly used spheroid-generating techniques due to both ease and reproducibility. This, combined with the enhanced image resolution attained by confocal microscopy compared to conventional wide-field approaches, allowed the identification and analysis of distinct morphological changes in migratory cells in a 3D environment following treatment with the migrastatic drug MI-192.

INTRODUCTION:

Three dimensional spheroid technologies for preclinical drug discovery and the development of potential cancer drugs are increasingly being favored over conventional drug screens; thus, there is more development of migrastatic – migration and invasion preventing – drugs. The rationale behind these developments in cancer treatment are clear: 3D spheroid assays represent a more

realistic approach for screening potential anti-cancer drugs as they mimic 3D tumor architecture more faithfully than cell monolayer cultures, recapitulate drug-tumor interactions (kinetics) more accurately, and allow the characterization of drug activity in a tumor-related setting. In addition, the rise of resistance to chemotoxic drugs in many cancer types and high death rates among cancer patients due to metastasis potentiated by the ability of cancer cells to migrate to distant tumor sites supports the inclusion of chemotherapeutic agents targeting the migratory potential of cancer cells as adjuvant treatment in future clinical cancer trials[1]. This is particularly the case in highly invasive cancers, such as high-grade glioblastomas (GBM). GBM management includes surgery, radiotherapy, and chemotherapy. However, even with combination treatment, most patients relapse within 1 year of initial diagnosis with a median survival of 11-15 months[2,3]. Huge advances in the field of 3D technology have been made over the last few years: rotative systems, microfabricated structures and 3D scaffolds, and other individual assays are being continually improved to allow routine testing on a large scale[4-7]. However, results obtained from these assays must be analyzed in a meaningful manner because data interpretation is often hindered by attempts to analyze 3D-generated data with 2D image analysis systems.

Despite being preferable in terms of image acquisition speed and reduced photo-toxicity, most wide-field systems remain limited by resolution[8]. Thus, apart from data read-outs relating to drug efficacy, detailed effects of drug action on 3D cellular structures of migrating cells are inevitably lost if imaged using a wide-field system. Conversely, confocal laser scanning microscopy (CLSM) captures high quality, optically sectioned images that can be reconstructed and rendered in 3D post-acquisition using computer software. Thus, CLSM is readily applicable to imaging complex 3D cellular structures, thereby enabling interrogation of the effects of anti-migrastatic inhibitors on 3D structures and in-depth analyses of cell migration mechanisms. This will undoubtedly guide future migrastatic drug development. Here, a combined workflow of spheroid generation, drug treatment, staining protocol, and characterization by high-resolution confocal microscopy is described.

PROTOCOL:

1. Generation of cell spheroids

Day 1

1.1 Prepare the standard culture medium as required by the cell line under investigation.

1.2 Carry out all tissue culture–associated steps in a tissue culture hood using sterile handling techniques.

1.3 Trypsinize and count cancer cells. Use 20 mL of cell suspension per plate. Keep the cell suspensions in clearly labeled sterile universal tubes.

1.4 Add a predetermined number of cells to each well. Both the initial number of cells and ultimate spheroid size required depends upon the proliferation rate of the cell line being

investigated.

NOTE: For established glioma cell lines such as U251 and KNS42[9,10], 5 x 10^3 cells/mL will produce a microscopically visible spheroid (200 or 800 μm) after 4 days of incubation.

1.5 Resuspend the cells in universal tubes by gentle inversion to avoid cell clumping. Pipette 200 μL of the cell suspension into each well of a 96-well plate. If all wells are not required, it is advisable to add 200 μL of 1x PBS to each empty well to avoid evaporation.

1.6 Incubate the cells in an incubator as normal at 37 °C.

NOTE: Cell lines such as glioma cancer cell lines will form spheroids within 24 h. Allow 3D cellular architecture to form by incubating the spheroid for 72 h.

Day 2

1.7 Check cells by bright-field microscopy after 24 h. Depending upon the cell line, cells may have formed a spheroid detectable in the bottom of the well.

NOTE: Established glioma cancer cell lines readily form spheroids within 24 h. Patient-derived glioma cancer cell lines may take up to 1-2 weeks.

2. Collagen invasion assay

Day 3

2.1 Place collagen, 5x culture medium, 1 M NaOH, and one 20 mL tube on ice.

2.2 Carefully and slowly add 10.4 mL of cold collagen into a chilled culture tube. Avoid bubbles. This quantity of collagen is enough for one 96-well plate. Upscaling is possible, but it is recommended that one 20 mL tube per plate is prepared at a time.

2.3 Gently add 1.52 mL of cold sterile 5x culture medium. Avoid bubbles.

2.4 Just before use, gently add 72 μL of cold sterile 1 M NaOH. Keep solution on ice.

2.5 Mix gently by pipetting. Avoid bubbles. Efficient mixing leads to a color change (from red to orange-red (pH 7.4) in the medium. Leave the mixture on ice until use.

2.6 Crucial step: Remove 190 μL of supernatant from the 96-well plate prepared on day 1. Be very careful not to disturb the spheroids that formed in the bottom of the well. Use the pipette at an angle towards the side, not the center, of the well.

2.7 Gently add 100 μL of the collagen mix to each well. To prevent any spheroid disturbance,

pipette the mix down the side of the well. Avoid bubbles. Keep any remaining collagen mix in the 20 mL tube at room temperature to assess polymerization.

2.8 Incubate plate in the incubator for at least 10 min to allow the collagen to polymerize. As a guideline, if the leftover collagen has set, becoming semisolid and sponge-like, the spheroids are ready to be treated with inhibitor.

2.9 Add the drugs or inhibitors at 2x concentration to the culture medium. Add the medium gently to each well (100 µL per well). Again, pipette the medium down the side of the well to avoid spheroid disturbance.

2.10 Observe and image each spheroid by bright-field microscopy at times T = 0 h, 24 h, 48 h, and 72 h to assess drug activity. Then return the plate to the incubator.

NOTE: Depending on the invasive behavior of the cell line, migration away from the original spheroid core may be observed from 24 h onwards.

3 Preparation of collagen embedded spheroids and migratory cells for confocal microscopy

3.1 Place the plate in a tissue culture hood and gently remove the supernatant (200 µL). Again, take care not to disturb the spheroid and avoid touching the collagen, as this may interfere with the collagen plug.

3.2 Replace the supernatant with 100 µL of 1x PBS. Repeat this wash step 3x.

3.3 Remove the final wash and replace with 4% formaldehyde in 1x PBS (100 µL per well).

CAUTION**:** Formaldehyde is a potential carcinogen. Handle with care in accordance with health and safety guidelines.

3.4 Place the 96-well plate on a lab bench, cover with foil, and leave for 24 h at room temperature.

3.5 Carefully remove the formaldehyde and replace with 1x PBS. Repeat this 1x PBS wash 3x.

3.6 Prepare 0.1% Triton X-100 in 1x PBS. Remove the 1x PBS wash and replace with 100 µL of the Triton X-100 solution. Incubate for 30 min at room temperature. In the meantime, prepare the blocking solution with 1x PBS and 0.05% skimmed milk powder and mix thoroughly.

3.7 Remove Triton X-100 and wash 3x with 1x PBS. Add 100 µL of blocking solution to each well and incubate for 15 min.

3.8 Dilute the required primary antibody in blocking buffer at the predetermined

concentration. Here, use anti-mouse IgG acetylated tubulin antibody (1:100).

3.9 Centrifuge the primary antibody-blocking buffer mix for 5 min at 15,682 x *g*. Carefully remove the blocking solution and add the supernatant (25−50 µL) to each well. Incubate in the dark at room temperature for 1 h.

3.10 Remove the antibody solution and wash 3x with 1x PBS (100 µL per well).

3.11 Dilute the secondary antibody in the blocking buffer at the recommended or predetermined concentration in addition to any additional fluorescent stains. Here, use 1:500 anti-mouse fluorophore-488 conjugated antibody, phalloidin-594 (1:500) for actin staining, and the DNA stain (DAPI).

3.12 Again, centrifuge the secondary antibody solution for 5 min at 13,000 rpm.

3.13 Remove the blocking solution from each well and add 25−50 µL of the secondary antibody/phalloidin/DAPI mix. Incubate in the dark for 1.5 h at room temperature.

3.14 Remove secondary antibody-dye solution and wash 3x with 1x PBS (100 µL per well).

3.15 Carefully lift individual collagen plugs by suction with a plastic pipette (200 µL) onto the center of a high-quality plain glass slide.

3.16 Add one drop of a suitable mountant to the collagen plug, ensuring the plug is completely covered. Avoid bubbles.

3.17 Apply coverslip of the optimal thickness for the microscope objective that will be used for imaging and allow to set overnight. Store the slides at room temperature in the dark.

4 Fluorescence microscopy

4.1 Capture fluorescent images using a suitable confocal microscope.

REPRESENTATIVE RESULTS:

Three-dimensional spheroid technology is advancing the understanding of drug-tumor interactions because it is more representative of the cancer-specific environment. The generation of spheroids can be achieved in several ways; low adherence 96-well plates were used in this protocol. After testing several products from different manufacturers, the plates used here were chosen because they consistently performed best in terms of successful spheroid production and uniformity. The replacement step, where the growth medium is replaced with the collagen matrix, is a critical point of the protocol; great care must be taken to remove most of the medium without disturbing the spheroid itself. Automated imaging for the characterization of drug-induced effects by wide-field microscopy may be considered to remove any handler bias, but currently commercially available instruments remain considerably more expensive than the

imaging approaches outlined here.

Wide-field epifluorescence microscopy allows examination of the effect of drug activity on cell migration and invasion. However, the resolution attained from wide-field microscopy is not good enough to allow detailed interpretation of results with regards to drug effect on cell morphology (**Figure 1**). Here, the preparation of glioma spheroids and migrating cells through easily reproducible staining protocols is described, followed by imaging using a confocal microscope. From the wide-field microscopy image analysis, it was evident that different morphological changes had occurred in the glioma cells following treatment with the MI-192 inhibitor, but clearly-defined details were lacking. Confocal microscopy confirmed the initial findings, and these higher resolution images allowed the assessment of the effect of MI-192 even further. Significant differences between untreated (control) spheroids, migrating cells (**Figure 2**), and treated spheroids and cells (**Figure 3**) became evident. Whereas the adult glioma cell line U251 appeared to migrate in 'spikes', radiating away from the original spheroid core with single cells detaching, the pediatric cell line KNS42 adopted a sheet-like migration pattern with few distinct cell spikes. Previously, different migration patterns among different cell lines (here the adult glioma cell line U251 and the pediatric cell line KNS42) were observed, potentially reflecting the cell type they arose from and site of tumor isolation. Crucially, an increase of acetylated tubulin with increasing inhibitor concentration (from 0.1−10 µM) was also uncovered, not only in the migrating cells, but also in the spheroid-associated cells. This was not evident in the initial wide-field microscopy acquired images. Further imaging would also allow the quantitative analysis of protein expression levels as a cellular response to treatment with migrastatic inhibitors.

In this study, it was demonstrated that the cells changed morphologically in response to treatment; cell rounding became apparent with increasing inhibitor concentrations and cell death at the highest inhibitor concentration (10 µM), with nuclear fragmentation evident in U251 cells and collapsed microtubules and nuclear fragmentation in KNS42. These findings are in keeping with previous observations that the anti-migratory activity of MI-192 on glioma cell lines is concentration dependent[13,14]. The overall workflow of the protocol is depicted in **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1. Spheroid cell invasion into collagen imaged by wide-field microscopy. Representative images of the cell lines U251 and KNS42 are shown after treatment with the inhibitor MI-192 at the anti-migratory concentration of 1 µM and at 24 h intervals. A control spheroid with no treatment is also shown. Potential anti- or pro-migratory effects are detected as highlighted (arrows). This is especially noticeable in KNS42 with seemingly no migration in either the control or treated spheroids. All images were taken at 4x. Scale bar = 1,000 µm. Scale bar in enlarged images for SF188 = 200 µm, KNS42 = 1,000 µm.

Figure 2. The effect of the migrastatic inhibitor MI-192 on glioma cell line U251 revealed by confocal microscopy. Fixed and stained glioma spheroids and migratory cells display distinct migratory phenotypes. Migratory cells close to the original spheroid edges are shown. U251 cell spheroids are characterized by spikes radiating away from the original spheroid with increasing cell rounding in cells apparent with increasing inhibitor concentration (arrow indicates cell spike).

Scale bar = 10 µm. Labels: red = actin, green = acetylated tubulin, blue = DAPI. For each image, a single representative optical section was captured with all settings with both pre- and post-image capture maintained for comparative purposes. All images were subsequently processed.

Figure 3. The effect of the migrastatic inhibitor MI-192 on the glioma cell line KNS42 revealed by confocal microscopy. KNS42 migration is characterized by sheetlike protrusions with single cell spikes (arrow indicates cell sheet). At the lowest inhibitor concentration this phenotype appears to be pronounced but is lost with increasing inhibitor concentration (scale bar = 10 µm); labels: red = actin, green = acetylated tubulin, blue = DAPI. For each image a single representative optical section was captured, with all settings with both pre- and post-image capture maintained for comparative purposes. All images were subsequently processed.

Figure 4. Summary of workflow. Incorporated in this workflow is the generation of spheroids, embedding in collagen, drug treatment, fixing, staining, and imaging by confocal microscopy.

DISCUSSION:

A novel way to create cancer cell spheroids for identification of migrastatic drug activity using high-resolution confocal microscopy is described. The use of low adherent plates over other techniques, such as hanging drops[15], has facilitated a means of generating reproducible and uniform spheroids for use in the collagen migration and invasion assays. The critical points in this protocol are the removal of growth medium from the 96-well plate prior to the cell spheroid embedding in a collagen matrix and the careful handling of the collagen plugs containing the spheroids thereafter. New technologies such as digital microfluidics[16] are now available and, although more expensive, could provide an alternative to the manual removal of media and fluids. The main limitation of the described protocol is that it is time-consuming when single spheroids are imaged and then analyzed manually by bright field microscopy in order to assess inhibitor drug activity. In addition, the reagents required for all steps of the process are expensive, and specialized equipment, namely a high-resolution confocal microscope, is also required. Optimal cell numbers required for seeding and the time taken to obtain a cell spheroid of the required size also must be predetermined prior to commencement of the collagen invasion assay.

The development of drugs targeting cancer cells, large-scale drug screening, and the characterization of drug activity for drug modification and improvement increasingly rely on 3D cell assays and technology. This protocol can be optimized and adapted for use with other experimental assays and numerous other cell types of importance in other disease systems. To date, the interpretation of data generated microscopically has been hampered by the application of wide-field microscopy, with images acquired offering limited resolution and plagued with inherent image blur resulting from light originating outside of the focal plane. The wide-field microscopy images shown here were acquired manually using an EVOS imaging system, a process that was time-consuming and could potentially introduce handler bias. New technologies, including automated workstations and analysis platforms[17,18], are now available but remain costly and are therefore still unavailable to many research laboratories. In addition, further software developments could aid in the analysis of high-resolution 3D rendered images to

accurately quantify phenotypic changes such as those noted here, and therefore the efficacy of inhibitors on cell migration. Furthermore, the application of super-resolution fluorescent imaging techniques that are increasingly becoming standard in many research laboratories will provide further insight into the migratory behavior and morphology of cells grown in 3D spheroid structures and treated with inhibitor drugs.

It was established that it is possible to fix and stain spheroids, following treatment with a migrastatic inhibitor, when embedded in collagen. This method was easy to perform, with all steps completed in 96-well plates, followed by mounting the collagen plugs containing the spheroids onto coverslips for imaging. In assessing the effect of inhibitor activity on cancer cell morphology, confocal microscopy was used to elucidate drug activity. Initial findings on the anti-migratory effect of inhibitor MI-192 from low-resolution images were confirmed by high-resolution confocal microscopy. This particular inhibitor targets histone deacetylase 3 (HDAC3). HDACs are enzymes involved in the epigenetic regulation of gene expression and have recently been of increasing interest as potential targets in cancer drug development. Previous experiences with MI-192 have shown that, at low concentrations, it regulates the acetylation of tubulin, leading to hyperacetylation and stabilization of microtubules. Stabilized microtubules are less dynamic, with a potential effect on migratory activities of cells. It was ascertained that the effect observed was present in both representative pediatric and adult glioma cell lines. A concentration-dependent increase in the tubulin acetylation status of both glioma cell lines was uncovered, a finding that has implications for the preselection of patients when considering complementary treatment with migrastatic drugs.

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DISCLOSURES:

The authors declare no conflict of interest.

REFERENCES:

1. Gandalovičová, A. et al. Migrastatics—anti-metastatic and anti-invasion drugs: promises and challenges. *Trends in Cancer*. **3** (6), 391-406 (2017).
2. Delgado-López, P. D., Corrales-García, E. M. Survival in glioblastoma: a review on the impact of treatment modalities. *Clinical and Translational Oncology*. **18**, 1062 (2016).
3. Foreman, P. M. et al. Oncolytic virotherapy for the treatment of malignant glioma. *Neurotherapeutics*. **14**, 333 (2017).
4. Rodrigues, T. et al. Emerging tumor spheroids technologies for 3D in vitro cancer modelling. *Pharmacology and Therapeutics Technologies*. **184**, 201-211 (2018).

5. Sirenko, O. et al. High-content assays for characterizing the viability and morphology of 3D cancer spheroid cultures. *Assay and Drug Development Technologies*. Mary Ann Liebert, Inc. **13** (7) (2015).
6. Wu, Q. et al. Bionic 3D spheroids biosensor chips for high-throughput and dynamic drug screening. *Biomedical Microdevices*. **20**, 82 (2018).
7. Mosaad, E., Chambers, K. F., Futrega, K., Clements, J. A., Doran, M. R. The microwell-mesh: a high-throughput 3D prostate cancer spheroid and drug-testing platform. *Scientific Reports* **8**, 253 (2018).
8. Jonkman, J. Brown, C. M. Any way you slice it—a comparison of confocal microscopy techniques*. Journal of Biomolecular Techniques*. **26** (2), 54–65 (2015).
9. Pontén J. Neoplastic human glia cells in culture. In: Fogh J., editor. *Human Tumor Cells in Vitro*. Boston, MA: Springer US. 175–206 (1975).
10. Takeshita I. et al. Characteristics of an established human glioma cell line, KNS-42. *Neurologica Medico Chirurgica*. **27** (7), 581-587 (1987).
11. Bance, B., Seetharaman, S., Leduc, C., Boëda, B., Etienne-Manneville, S. Microtubule acetylation but not detyrosination promotes focal adhesion dynamics and cell migration*. Journal of Cell Science*. **132** (2019).
12. Bacon, T. et al. Histone deacetylase 3 indirectly modulates tubulin acetylation. *Biochemical Journal.* **472**, 367-377 (2015).
13. Jackman, L. et al. Tackling infiltration in paediatric glioma using histone deacetylase inhibitors, a promising approach*. Neuro-Oncology*. **20** (suppl_1), i19-i19 (2018).
14. Bhandal, K. et al. Targeting glioma migration with the histone deacetylase inhibitor MI192. *Neuro-Oncology.* **19** (suppl_1), i12-i12 (2017).
15. Del Duca, D., Werbowetski-Ogilvie, T. E., Del Maestro, R. Spheroid preparation from hanging drops: characterization of a model of brain tumor invasion. *Journal of Neuro-oncology*. **67** (3), 295-303 (2004).
16. Bender, B. F., Aijian, A. P., Garrell, R. L. Digital microfluidics for spheroid-based invasion assays. *Lab on a Chip*. **16** (8), 1505-1513 (2016).
17. Vinci, M. et al. Advances in establishment and analysis of three dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biology*. **10**, 29 (2012).
18. Härmä, V. et al. Quantification of dynamic morphological drug responses in 3D organotypic cell cultures by automated image analysis. *PLOS ONE*. **9** (5), e96426 (2014).