The Influence of Human Astrocyte-Conditioned Media on Glioblastoma Multiforme Response to Temozolomide and Bay 11-7082

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ABSTRACT Glioblastoma Multiforme (GBM) cells interact with a complex, heterogeneous tumor microenvironment (TME). This TME consists of astrocytes, endothelial cells, microglia, and pericytes, which together play a role in GBM progression and resistance. However, there are not enough in vitro three-dimensional (3D) models to study the effect of the TME on GBM resistance to chemotherapeutics. In this study, we created a GBM TME by culturing GBM cells with media that had been conditioned by human astrocytes (HA) in 3D microwells. In order to investigate the effect of the TME on GBM resistance to chemotherapeutic agents, cells were treated with Temozolomide (TMZ) in combination with nuclear factor-κB (NF-κB) inhibitor “Bay 11-7082”. We examined the influence of HA conditioned media (CM) on the expression of various genes and the response to TMZ and Bay 11-7082 in our 3D cultures. Our data suggested that proteins and metabolic factors produced by HA in CM can significantly alter GBM response to chemotherapeutics. Our results indicated lower levels of apoptosis- and drug resistance-related genes were detected in LN229 and U87 cultures in their respective cell culture media compared to HA CM. Our results confirmed HA affect GBM response to therapy.

INDEX TERMS Glioblastoma, 3D models, tumor microenvironment, co-culture.

I. INTRODUCTION Glioblastoma Multiforme (GBM) has the highest mortality among adult primary brain tumors [1]. GBM patients have an ~30% survival rate over one year, and only ~3–5% of patients survive beyond 5 years [2], [3]. Following diagnosis, patients undergo maximal safe surgical resection followed by radiotherapy and concomitant oral chemotherapy using the DNA-alkylating agent Temozolomide (TMZ) [4], [5]. Although recent treatment methods have increased the survival rate, the overall clinical outcome remains unsatisfactory, in part due to rising GBM resistance to TMZ [2]. The main factor believed to influence this chemoresistance is the O 6-methylguanine-DNA methyltransferase (MGMT) gene [6]. However, the molecular mechanism of chemoresistance to TMZ is more complex than a simple dependence on one gene. For instance, MGMT expression can be suppressed by the nuclear factor-κB (NF-κB) inhibitor Bay 11-7082. Subsequently, combined treatment of GBM with Bay 11-7082 and TMZ can be a promising method to overcome chemoresistance to TMZ [7]–[9].

The tumor microenvironment (TME), including the extra-cellular matrix and various stromal cells, (e.g., astrocytes and endothelial cells (EC)) regulates GBM development and progression [10], [11]. Astrocytes, unique to the central nervous system (CNS), can comprise ~50-90% of all brain cells (depending on the brain region) and typically play a neuroprotective role [12]. Astrocytes become reactive under pathological conditions, and are characterized by increased expression of glial fibrillary acidic protein (GFAP) and vimentin [13], [14]. These reactive astrocytes can protect GBM cells from cytotoxic chemotherapy agents [15]. Drug screening studies require an in vitro model to recapitulate the in vivo TME biology [16]. Therefore, culturing cells in a three-dimensional (3D) system to mimic tissue structure in the in vivo environment is more ideal...
than the traditional two-dimensional (2D) culture method. In conventional 2D monolayers, several in vivo extracellular matrix components are lost, including cell-to-cell and cell-to-matrix interactions that are crucial for differentiation, proliferation, vitality, drug metabolism, gene expression, and protein synthesis [17]–[20].

The role of the TME in drug screening has been limited by a lack of 2D models that accurately mimic the human brain microenvironment. Subsequently, anticancer drugs efficacy and/or cytotoxicity studies often show misleading drug screening results. The drug screening process can be improved by recreating the TME physiological environment in new 3D models, particularly for GBM. Previous studies indicated that 3D tumor spheroids are a promising in vitro model due to the enhanced cellular interactions via adhesion and secretion of soluble factors of the tumor which mimic the in vivo TME and GBM response [16], [21].

In the current study, we investigated the effect of the TME on GBM growth and protein expression following treatment with NF-κB inhibitor, Bay 11-7082, and an alkylating agent, TMZ. We utilized our previously optimized and fabricated poly(ethylene glycol) dimethyl acrylate (PEGDA) hydrogel microwells [22], [23] and cultured LN229 and U87 cells in standard media or media that had been conditioned (CM) by human astrocytes (HA).

II. METHODS
A. MICROWELL FABRICATION
PEGDA microwells were fabricated as previously reported [22], [24], [25]. In brief, 25 × 25 mm cover glass slides were washed with sodium hydroxide and treated with 3-(trimethoxysilyl) propyl methacrylate 98% (TMSPMA, CAT#440159, Life Technologies, New York, NY, USA) to enhance the hydrogel attachment to the cover glass. To prepare the hydrogel solution, 40% (w/v) of PEGDA700 (CAT#455008, Life Technologies) and 0.2% (w/v) of the photoinitiator (PI) 2-hydroxy-2-methyl propiophenone was dissolved in Phosphate Buffered Saline (PBS, CAT#45000, Life Technologies) and 0.2% (w/v) of the photoinitiator (PI) 2-hydroxy-2-methyl propiophenone was dissolved in Phosphate Buffered Saline (PBS, CAT#45000, Life Technologies). Treated slides were covered with 20 µl of the hydrogel solution and exposed to Lumen Dynamics OmniCure® Series 2000 (Lumen Dynamics Group Inc, Mississauga, ON, Canada) for 36 s at a working distance of 6 inches. After polymerization of the first layer, 300 µl of the hydrogel solution was added to the slide and exposed to UV light for another 36 s with the desired photomask on top of it. The photomask was designed with AutoCAD (Autodesk Inc, San Rafael, CA, USA) in a round pattern of 400 µm in diameter and purchased from CAD/Art Services Inc (Bandon, OR, USA).

B. CELL LINES AND CULTURE
Glioblastoma cell lines LN229 and U87 were purchased from the American Tissue Culture Collection (CAT#CRL2611, CAT#HTB14, respectively, ATCC, Manassas, VA, USA). GBM cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, CAT#45000-304, Corning, New York, NY, USA) supplemented with 10% (v/v) of fetal bovine serum (FBS, CAT#TMS-013-B, VWR, Radnor, PA, USA) and 1% (v/v) penicillin/streptomycin (CAT#97063-708, VWR). The LN229 and U87 cells were subjected to 10–15 passages after purchase and reached approximately 80% confluency prior to seeding in the microwells. Primary HA were purchased from ScienCell (CAT#1800, Carlsbad, CA, USA), and were grown in the basal medium supplemented with 2% (v/v) fetal bovine serum, 1% (v/v) astrocyte growth supplement, and 1% (v/v) antibiotic solution (CAT#1801, ScienCell). HA were subjected to 2-5 passages and reached approximately 80% confluency prior to seeding in the microwells. All cells were maintained under a sterile tissue culture hood and kept in a 95% air-5% CO₂ humidified cell incubator at 37 °C. To form 3D spheroids, LN229 or U87 cells were trypsinized and cultured in PEGDA microwells with a density of 0.2 × 10⁶ cells/ml. 3D spheroid formation inside of the microwells was monitored using an Olympus fluorescence microscope (Tokyo, Japan).

C. CONDITIONED MEDIA
HA were cultured separately until 70% confluency in serum-free basal medium. The CM was collected 48 hours later and centrifuged at 1000 g for 10 min to remove debris. This CM was then used to culture LN229 and U87 cells in HA CM.

D. DRUG ADMINISTRATION
Bay 11-7082 and/or TMZ was introduced to 3D spheroids on day 7. Bay 11-7082 (CAT#B5556, Sigma-Aldrich, St.Louis, MO) was dissolved in dimethylsulfoxide (DMSO, CAT#sc-358801, Santa Cruz Biotechnology, Dallas, TX, USA) to get 50 mM stock solution and diluted further to 10 µM using cell culture medium [26], [27]. In order to dissolve Temozolomide (TMZ, CAT#T2577, Thermo Fisher Scientific, Waltham, MA, USA), DMSO was also used to get 50 mM of stock solution concentration and diluted to 600 µM using cell culture medium. Spheroids were kept for 7 additional days after one-time drug(s) administration. The control (untreated) group was kept under the same conditions as treatment groups using cell culture media. The DMSO final concentration in treated sample’s cell culture media was 0.1%, which has previously been shown to not negatively affect cell viability [28].

E. QUANTIFICATION OF CELL VIABILITY
To quantify cell viability, treated and untreated spheroids were collected from the microwells in separate centrifuge tubes and centrifuged at 180 g for 3 min to isolate them from cell culture medium, then washed with PBS once, and dissociated into single cells with trypsin. The single cells from each sample were resuspended in the cell culture medium, stained with 0.4% Trypan blue solution (CAT#15250061, Thermo Fisher Scientific), and then counted using a hemocytometer. The viability of the cells in each treatment and media group was normalized to their respective untreated group.
TABLE 1. Primer sequences used in qPCR.

| Gene  | Forward Primer | Reverse Primer |
|-------|----------------|----------------|
| Bcl-2 | CCCCCGCATTGTTCGTTTAT | CAGTCTACTTCTTCTTGAGT |
| Bax   | GCCCTTTGTCCTATCCTTT | CATCCTCTGCAGCTTCTGCT |
| NF-κB | TGGGCGACACAAGTTC | TGAATAGTGGGAGGCTTTG |
| MGMT  | CCGTTGCGGACTTTTGCT | CCCCCTGGCCAGGGCTTTA |
| β-actin | CACCCATGGCAATGAC | AGGTCTTGGCAGATGTC |

F. GENE EXPRESSION ANALYSIS BY QUANTITATIVE PCR (qPCR)

To quantify gene expression, treated and untreated spheroids were collected from the microwells and centrifuged at 180 g for 3 min, washed once with PBS, and then trypsinized to dissociate the spheroid into single cells. Total RNA was extracted using a RNeasy Mini Kit (CAT#79254, Qiagen Germantown, MD, USA) according to the manufacturer’s instructions. A Nanodrop 2000 series (Thermo Fisher Scientific) was used to quantify extracted RNA using the optical density (OD) at 260 and 280 nm. High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (CAT#4374966, Thermo Fisher Scientific) was used to synthesize cDNA from RNA samples. Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). In brief, 20 µL of reaction mixture, containing 10 µL of PerfeCTa SYBR Green SuperMix Reaction Mixes (CAT#AB4323A, Quanta bio, Beverly, MA, USA), 300 nM primers, and 50 ng cDNA was added to the qPCR micro-well plate. The thermal cycling sequence was programmed for an initial incubation at 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 53 °C for 45 s. Bcl-2, Bax, NF-κB p65, and MGMT expression was investigated using the respective primer sequences listed in Table 1. Target gene expression was normalized to β-actin levels in the same reaction using the ΔΔCt method [29].

G. WESTERN BLOT

Treated and untreated spheroids were collected from the microwells, washed twice with cold PBS, and dissociated with trypsin into single cells. Radio immunoprecipitation assay buffer (RIPA buffer) with phosphatase inhibitor (CAT#89900, Thermo Fisher Scientific) was used to lyse the single cells. Cell lysates were incubated on ice for 20 min and centrifuged at 4 °C at 220 g for 10 min. The supernatants were collected, and the concentration of protein was measured using a micro BCA protein assay kit (CAT#PI23235, VWR). Equal amounts (15 µg) of proteins were loaded to 12% Mini-PROTEAN TGX Gel (CAT#4561046, Bio-Rad, Hercules, CA, USA) and transferred to a PVDF membrane (CAT#1620177, Bio-Rad). Membranes were blocked with 3% milk (in 1X TBS-Tween20) for 1 h, followed by primary antibody incubation overnight at 4°C. Protein bands were visualized by applying Clarity Western ECL Substrate (CAT#1705060, Bio-Rad) to the membrane and imaging by ECL Western blot detection system (Amersham Pharmacia Biotech). The data were normalized to β-actin levels. Bcl-2 (CAT#sc-7382, Santa Cruz, Dallas, TX, USA), NF-κB p65, MGMT, Bax and β-Actin (CAT#ab6276, CAT#ab16502, CAT#ab108630, CAT#ab32503, respectively, Abcam, Cambridge, MA, USA) antibodies were used at a concentration of 1:1000. Goat anti-Mouse IgG (H+L) secondary antibody (HRP) and Goat anti-Rabbit IgG (H+L) secondary antibody (HRP) (CAT#NB7539, CAT#NB7183, respectively, Novus Biologicals, Centennial, CO, USA) were used at 1:2000 dilution.

H. STATISTICAL ANALYSIS

All results were derived from three independent experiments performed in triplicate. Viability and differences between culture methods within treatment groups was analyzed using a 2-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test. All statistical analyses were conducted using GraphPad Prism (v9) with a significance level of 0.05. Data is presented as mean ± standard error of the mean (SEM).

III. RESULTS

A. CONDITIONED MEDIA (CM) IN CULTURE IMPACTS TREATMENT RESPONSE

We have used 3D microwells for GBM studies to understand the effect of the TME on GBM growth [22], [23], as well as to investigate the interaction of 3D GBM spheroids with endothelial cells [30]. Recently, we studied the effect of TMZ and/or NF-κB inhibitor (Bay 11-7082) on the interaction of 3D GBM spheroids with astrocytes [24], [31]. Astrocytes are known to produce cytokines and growth factors that modulate blood-brain barrier (BBB) properties in the brain vascular endothelium. We hypothesized the presence of astrocytes in GBM culture may enhance the drug sensitivity. Therefore, we examined the influence of HA conditioned media (CM) on the expression of factors to study 3D GBM response to TMZ and Bay 11-7082 (Fig. 1a-d).

Cell viability was assessed 7 days after drug administration and normalized to control (i.e., untreated) groups (Fig. 1e). Viability was analyzed using a 2-way ANOVA with Tukey’s multiple comparisons test. The ANOVA revealed a significant effect of drug (p<0.0001) and culture method (p<0.0001) on cell viability. Treatment of spheroids with Bay 11-7082 or TMZ significantly reduced cell viability in LN229 and U87 cultures using standard media (p<0.05). However, this effect was less pronounced in spheroids cultured in CM. U87 cells cultured in standard media were significantly less viable than U87 cells cultured in HA CM. The effect of HA CM on viability was most pronounced in cultures treated with both Bay 11-7082 and TMZ. Viability of LN229+HA CM
was significantly higher than LN229 cells in standard media (p<0.001) as well as U87+HA CM cells compared to their standard media counterparts (p<0.001).

**B. THE INFLUENCE OF HA CM ON APOPTOSIS AND TMZ RESISTANCE IN GBM**

To understand how astrocytes affect the GBM response to Bay 11-7082 and/or TMZ treatment, we investigated the expression of apoptosis- and drug resistance-related genes. Our results indicated that Bcl-2, Bax, NF-κB-p65, and MGMT genes were downregulated after Bay 11-7082 and/or TMZ treatments (Fig. 2 and Table 2). Treatment with Bay 11-7082 and/or TMZ significantly decreased gene expression for Bcl-2, Bax, NF-κB, and MGMT. LN229 cells cultured in HA CM had significantly higher Bcl-2 expression following treatment with Bay 11-7082 and co-treatment with Bay 11-7082 and TMZ (p<0.05, Figure 2c). Additionally, U87 cells treated with both Bay 11-7082 and TMZ had significantly higher Bcl-2 expression differences between media types (p<0.05, Figure 2a). There were no significant differences in Bax expression of LN229 or U87 cells between media types (Figure 2b). NF-κB-p65 gene expression was higher in cells cultured in HA CM and co-treated with both Bay 11-7082 and TMZ (p<0.05, Figure 2c). Lastly, LN229 cells cultured in HA CM had significantly higher MGMT expression following co-treatment with Bay 11-7082 and TMZ (p<0.01, Figure 2d). Western blot results confirmed the changes in Bcl-2, Bax, NF-κB-p65, and MGMT gene expression (Fig. 3). Together, our gene expression and western blot results suggest the presence of astrocytes alter apoptosis-related genes in GBM and the subsequent response to co-treatment with Bay 11-7082 and TMZ.

**IV. DISCUSSION**

3D culture models have been shown to better recapitulate the GBM TME, and are thus considered to be more accurate for drug screening compared to 2D culture systems. Additionally, cancer cell lines exhibit different gene expression in 3D compared to 2D. Various genes responsible for proliferation, chemosensitivity, angiogenesis, and invasion observed in 3D systems are closer to in vivo conditions [20], [32]–[38]. Additionally, interactions between GBM, HA, and human brain microvascular endothelial cells (HBMEC) are crucial in GBM proliferation and response to therapy treatment. We used our cost-effective and timesaving PEGDA hydrogel microwells [22], [25] to generate 3D spheroids and perform
TABLE 2. Average gene expression fold changes in cultures with traditional media and HA CM, normalized to the untreated groups, respectively. qRT-PCR and the ΔΔ Ct formula was used to quantitate expression levels.

|        | Samples | Drug 1 | Drug 2 | Drug 1 + Drug 2 |
|--------|---------|--------|--------|-----------------|
| Bcl-2  | LN229   | 0.54 ± 0.11 | 0.65 ± 0.12 | 0.52 ± 0.09 |
|        | LN229 + HA CM | 0.77 ± 0.17 | 0.80 ± 0.15 | 0.74 ± 0.15 |
|        | U87     | 0.56 ± 0.13 | 0.62 ± 0.11 | 0.48 ± 0.08 |
|        | U87 + HA CM | 0.68 ± 0.12 | 0.71 ± 0.13 | 0.67 ± 0.09 |
| Bax    | LN229   | 0.56 ± 0.18 | 0.72 ± 0.13 | 0.66 ± 0.15 |
|        | LN229 + HA CM | 0.61 ± 0.21 | 0.69 ± 0.13 | 0.86 ± 0.20 |
|        | U87     | 0.48 ± 0.13 | 0.70 ± 0.12 | 0.57 ± 0.12 |
|        | U87 + HA CM | 0.52 ± 0.11 | 0.62 ± 0.19 | 0.62 ± 0.11 |
| NF-κB-p65 | LN229   | 0.53 ± 0.15 | 0.59 ± 0.12 | 0.28 ± 0.14 |
|        | LN229 + HA CM | 0.65 ± 0.20 | 0.73 ± 0.22 | 0.52 ± 0.14 |
|        | U87     | 0.62 ± 0.14 | 0.63 ± 0.15 | 0.38 ± 0.10 |
|        | U87 + HA CM | 0.77 ± 0.14 | 0.72 ± 0.17 | 0.66 ± 0.14 |
| MGMT   | LN229   | 0.52 ± 0.18 | 0.67 ± 0.14 | 0.36 ± 0.09 |
|        | LN229 + HA CM | 0.68 ± 0.15 | 0.72 ± 0.14 | 0.66 ± 0.13 |
|        | U87     | 0.48 ± 0.13 | 0.66 ± 0.15 | 0.51 ± 0.14 |
|        | U87 + HA CM | 0.65 ± 0.13 | 0.74 ± 0.13 | 0.63 ± 0.16 |

In summary, we assessed GBM response to test the sensitivity of GBM cell lines LN229 & U87 to the monotherapy and combined treatment of Bay 11-7082 and TMZ in HA CM. Our results suggest that the GBM TME is influenced by the presence of astrocytes and can significantly alter GBM response to treatment.

V. CONCLUSION

In this study, we examined the role of the TME on GBM growth, including HA CM on the response to Bay 11-7082 and/or TMZ using PEGDA hydrogel microwells. However, this study did have some limitations since our static model takes advantage of the hydrophobicity of PEGDA, which is not in healthy brain TME. Furthermore, although this model utilizes three cells involved in the BBB, the cells were allowed to self-aggregate and may not fully recapitulate the in vivo TME architecture. Future models can include various ratios of additional neural cells (e.g., HA or HBMEC) in order to recapitulate the in vivo TME of GBM. The pathophysiology of GBM is quite complicated and it has to be expected that a single drug will not be able to resolve the problem by targeting one particular molecular mechanism. Therefore, future studies should include high-throughput screening and personalized medicine approaches. Additionally, this model can be used to ascertain the role of the TME to investigate apoptosis pathways after drug treatment, or other drugs combination (e.g., Bevacizumab, Bay 11-7082, and TMZ).
REFERENCES

[1] B. G. Harder, M. R. Blomquist, J. Wang, A. J. Kin, G. F. Woodworth, J. A. Winkles, J. C. Loftus, and N. L. Tran, “Developments in blood-brain barrier penetration and drug repurposing for improved treatment of glioblastoma,” *Frontiers Oncol.*, vol. 8, p. 462, Oct. 2018.

[2] W. P. Mason, “Emerging drugs for malignant glioma,” *Expert Opinion Emerg. Drugs*, vol. 13, no. 1, pp. 81–94, Mar. 2008.

[3] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, “Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries,” *CA, Cancer J. Clin.*, vol. 68, no. 6, pp. 394–424, 2018.

[4] R. Stupp et al., “Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial,” *Lancet Oncol.*, vol. 10, no. 5, pp. 459–466, May 2009.

[5] R. Stupp et al., “Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma,” *New England J. Med.*, vol. 352, no. 10, pp. 987–996, 2005.

[6] N. Auger, J. Thillet, K. Wanherdick, A. Idhiba, M.-E. Legrier, B. Dutrillaux, M. Sanson, and M.-F. Poupot, “Genetic alterations associated with acquired temozolomide resistance in SNU-19, a human glioma cell line,” *Mol. Cancer Therapeutics*, vol. 5, no. 4, pp. 2182–2192, Sep. 2006.

[7] X. Wang, L. Jia, X. Jin, Q. Liu, W. Cao, X. Gao, M. Yang, and B. Sun, “NF-κB inhibitor reverses temozolomide resistance in human glioma TRU251 cells,” *Oncol. Lett.*, vol. 9, no. 6, pp. 2586–2590, Jun. 2015.

[8] A. Kumar, Y. Takada, A. Boriek, and B. Aggarwal, “Nuclear factor-κB: Its role in health and disease,” *J. Mol. Med.*, vol. 82, no. 7, pp. 434–448, Jul. 2004.

[9] J. Litak, C. Grochowski, J. Litak, I. Osuchowska, K. Gosik, E. Radzikowska, P. Kamieniak, and J. Rolinski, “TLR-4 signaling vs. immune checkpoints, miRNAs molecules, cancer stem cells, and wingless-signaling interplay in glioblastoma multicellular—Future perspectives,” *Int. J. Mol. Sci.*, vol. 21, no. 9, p. 3114, Apr. 2020, doi: 10.3390/ijms21093114.

[10] D. F. Quail and J. W. Rice, “The microenvironmental landscape of brain tumors,” *Cancer Cell*, vol. 31, no. 3, pp. 326–341, Mar. 2017.

[11] T. Simon, E. Jackson, and G. Giamas, “Breaking through the glioblastoma micro-environment via extracellular vesicles,” *Oncogene*, vol. 39, no. 23, pp. 4477–4490, Jun. 2020, doi: 10.1038/s41388-020-1308-2.

[12] A. Øren, “Production of neuroprotective NGF in astrocyte–T helper cell cocultures is upregulated following antigen recognition,” *J. Neuroimmunol.*, vol. 149, nos. 1–2, pp. 59–65, Apr. 2004, doi: 10.1016/j.jneuroim.2003.12.009.

[13] D. A. Crooks, C. L. Scholtz, G. Vowles, S. Greenwald, and S. Evans, “The glial reaction in closed head injuries,” *Neuropathol. Appl. Neurobiol.*, vol. 20, no. 6, pp. 629–638, Jun. 2002, doi: 10.1046/j.1469-7580.2002.00064.x.

[14] H. van de Waterbeemd and E. Gifford, “ADMET in silico modelling: Towards prediction paradise?” *Nature Rev. Drug Discovery*, vol. 2, no. 3, pp. 192–204, Mar. 2003.

[15] J. Saji Joseph, S. Tebogo Malindisa, and M. Ntwasa, “Two-dimensional (2D) and three-dimensional (3D) cell culturing in drug discovery,” in *Cell Culture*, London, U.K.: IntechOpen, 2019.

[16] J. Lee, M. J. Cuddihy, and N. A. Kotov, “Three-dimensional cell culture matrices: State of the art,” *Tissue Eng. B Rev.*, vol. 14, no. 1, pp. 61–86, Mar. 2008.

[17] M. Zietarska, C. M. Maugar, A. Filiali-Mouhim, M. Alam-Fahmy, P. N. Tonin, D. M. Provencher, and A.-M. Mes-Masson, “Molecular description of a 3D in vitro model for the study of epithelial ovarian cancer (OVAR-3),” *Mol. Cancer Res.*, vol. 7, no. 10, pp. 1167–1178, Oct. 2009.

[18] K. Shield, M. L. Ackland, N. Ahmed, and G. E. Rice, “Multicellular spheroids in ovarian cancer metastasis: Biology and pathology,” *Gynecol. Oncol.*, vol. 113, no. 1, pp. 143–148, Apr. 2009.

[19] L. A. Gurski, N. J. Petrelli, X. Jia, and M. C. Farach-Carson, “3D matrices for anti-cancer drug testing and development,” *Oncol. Issues*, vol. 25, pp. 20–25, Jan./Feb. 2010.

[20] S. Ghosh, G. C. Spagnoli, I. Martin, S. Ploege, P. Demougin, M. Hebar, and A. Reschner, “Three-dimensional culture of melanoma cells profoundly affects gene expression profile: A high density oligonucleotide array study,” *J. Cellular Physiol.*, vol. 204, no. 2, pp. 522–531, 2005.

[21] I. M. Ghobrial, T. E. Witzig, and A. A. Adjei, “Targeting apoptosis pathways in cancer therapy,” *CA Cancer J. Clin.*, vol. 55, no. 3, pp. 94–178, May/Jun. 2005, doi: 10.3322/canjclin.55.3.178.

[22] J. M. Hardwick and L. Soane, “Multiple functions of BCL-2 family members in cancer,” *Cold Spring Harbor Perspect. Biol.*, vol. 5, no. 2, Feb. 2013, Art. no. a008722, doi: 10.1101/cshperspect.a008722.

[23] S. A. Valdés-Rives, D. Casique-Aguirre, L. Germán-Castellán, M. A. Velasco-Velázquez, and A. González-Arenas, “Apostotic signaling pathways in glioblastoma and therapeutic implications,” *BioMed Res. Int.*, vol. 2017, pp. 1–12, Oct. 2017.

[24] N. D. Perkins, “Integrating cell-signalling pathways with NF-κB and IKK function,” *Nature Rev. Mol. Cell Biol.*, vol. 8, no. 1, pp. 49–62, Jan. 2007, doi: 10.1038/nrm2083.
X. Zhang, “Resveratrol reverses temozolomide resistance by downregulation of MGMT in T98G glioblastoma cells by the NF-κB-dependent pathway,” Oncol. Rep., vol. 27, no. 6, pp. 2050–2056, Mar. 2012, doi: 10.3892/or.2012.1715.

T. Borovski, P. Beke, O. van Tellingen, H. M. Rodermond, J. J. Verhoeuff, V. Lascano, J. B. Daalhuisen, J. P. Medema, and M. R. Sprick, “Therapy-resistant tumor microvascular endothelial cells contribute to treatment failure in glioblastoma multiforme,” Oncogene, vol. 32, no. 12, pp. 1539–1548, Mar. 2013, doi: 10.1038/onc.2012.172.

S. Tiwary, J. E. Morales, S. C. Kwiatkowski, F. F. Lang, G. Rao, and J. H. McCarty, “Metastatic brain tumors disrupt the blood-brain barrier and alter lipid metabolism by inhibiting expression of the endothelial cell fatty acid transporter Mfsd2a,” Sci. Rep., vol. 8, no. 1, p. 8267, May 2018, doi: 10.1038/s41598-018-26636-6.

R. K. Jain, E. Di Tomaso, D. G. Duda, J. S. Loeffler, A. G. Sorensen, and T. T. Batchelor, “Angiogenesis in brain tumours,” Nature Rev. Neurosci., vol. 8, no. 8, pp. 610–622, 2007.

E. C. LaCasse, S. Baird, R. G. Korneluk, and A. E. MacKenzie, “The inhibitors of apoptosis (IAPs) and their emerging role in cancer,” Oncogene, vol. 17, no. 25, pp. 3247–3259, Dec. 1998, doi: 10.1089/sj/onc.1202569.

F. F. Angileri, H. Aguennouz, A. Conti, D. La Torre, S. Cardali, R. Crupi, C. Tomasello, A. Germanò, G. Vita, and F. Tomasello, “Nuclear factor-κB activation and differential expression of Survivin and Bcl-2 in human grade 2–4 astrocytomas,” Cancer, vol. 112, no. 10, pp. 2258–2266, May 2008, doi: 10.1002/cncr.23407.

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