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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 extracellular 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

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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/w) 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). Treated slides were covered with 20 μl of the hydrogel solution and exposed to Lumen Dynamics the 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% CO_2 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 0.2×10^6 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	CCCCGCGACTCCTGATTCAT	CAGTCTACTTCCTCTGTGATGTTGT
Bax	GCCCTTTTGCTTCAGGGTTTC	CATCCTCTGCAGCTCCATGT
NF-κB	TGCCCAGCACAGAGGTG	TGAAATAGGTGGGGACGCTGT
MGMT	CCGTTTGCGACTTGGTACTTGG	CCCCTTGCCCAGGAGCTTTA
β-actin	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGATGTCCACGT

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 $\Delta\Delta$Ct 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

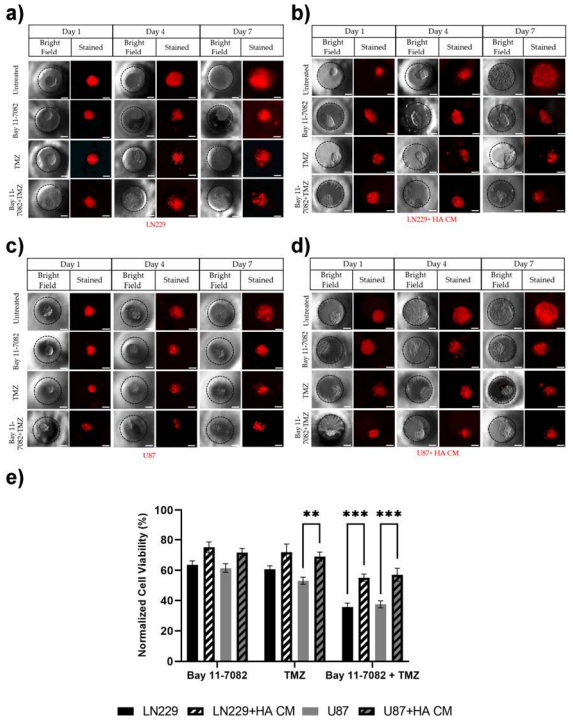

FIGURE 1. Representative images of cultured cells in the PEGDA microwells (400 μm in diameter) after 7 days of drug administrations with Bay 11-7082, TMZ, and Bay 11-7082 + TMZ or control. Images were obtained on Day 1, Day 4, and Day 7 to observe the disaggregation of the dead cells from the spheroids. LN229 and U87 cells were stained with cell tracker red. Dotted black lines represent the edges of the microwells. 20X objective. Scale bars are 100 μm. (a) LN229 cultures, (b) LN229 cultures with HA CM, (c) U87 cultures, (d) U87 cultures with HA CM. (e) Bar graph shows mean ± SEM of cell viability of LN229 and U87 cultured with and without HA CM. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

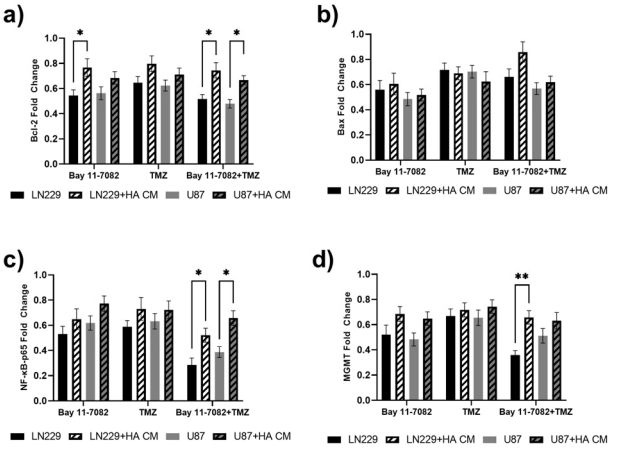

FIGURE 2. Gene expression analysis. Fold changes of (a) Bcl-2, (b) Bax, (c) NF-κB-p65, and (d) MGMT genes in GBM cultures with and without HA CM. 3D spheroids were treated with or without drugs for 7 days and digested to single cells for quantitative PCR experiments. Results were normalized to β-actin total RNA level in untreated groups. Asterisks on single bars represent significant differences compared to untreated group. * indicates $p < 0.05$, ** indicates $p < 0.01$.

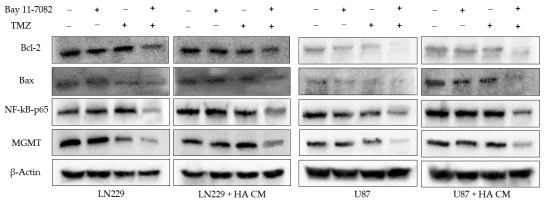

FIGURE 3. Western blot analysis. Levels of Bcl-2, Bax, NF-κB-p65, and MGMT were examined in LN229 and U87 cultures with and without HA CM on day 7 after treatment with Bay 11-7082 and/or TMZ.

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 2a). Additionally, U87 cells treated with both Bay 11-7082 and TMZ had significant 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.

Bcl-2			
Samples	**Bay 11-7082**	**TMZ**	**Bay 11-7082 + TMZ**
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			
Samples	**Bay 11-7082**	**TMZ**	**Bay 11-7082 + TMZ**
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			
Samples	**Bay 11-7082**	**TMZ**	**Bay 11-7082 + TMZ**
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			
Samples	**Bay 11-7082**	**TMZ**	**Bay 11-7082 + TMZ**
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

drug screening using GBM cell lines LN229 and U87. Tumor cells formed 3D spheroids at the bottom of each microwell 7 days after cell seeding. To assess whether the drug response was due to TME recapitulation in the microwells, we cultured LN229 and U87 in standard and HA CM. We investigated the response of GBM cultures to NF-κB inhibitor, Bay 11-7082, and an alkylating agent, TMZ. Cells cultured in CM had lower viability on day 7 after Bay 11-7082 and/or TMZ administration. Our results showed that cells cultured in CM were more resistant to the combined drug treatment. Our results suggested HA CM altered GBM survival in response to treatment in comparison to standard media (i.e., unconditioned) alone.

The interaction within the GBM tumor progression and resistance to drugs remains unclear. Therefore, we investigated the influence of astrocytes on GBM response to drug treatment. The apoptosis signaling pathway is an essential mechanism to maintain the balance between cell proliferation and cell death in GBM [39]. The B-cell lymphoma-2 (Bbl-2) protein family which includes Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, Bfl-1/A1, Bcl-B, Bax, Bak, and Bok, regulates the apoptosis pathway [40]. Bcl-2 is an anti-apoptotic protein and controls the mitochondrial membrane permeability, which can inhibit the apoptosis process; whereas Bax is pro-apoptotic protein [41]. Also, NF-κB which is a transcription factor for a large group of genes, involves in apoptosis [42]. Previous studies have demonstrated that NF-κB expression directly regulates MGMT expression which is known as a DNA repair enzyme and can induce chemoresistance in GBM cells [43]. Our results showed downregulation of NF-κB and MGMT after combined treatment compared to Bay 11-7082 or TMZ alone.

HBMECs are part of the BBB and provide protection for GBM cells against TMZ treatment while promoting the expansion and survival of GBM cells [44]. The selective permeability property of the BBB limits the penetration of therapeutic agents to the TME in the brain [45]. HBMECs and GBM cell interactions involve EC-intrinsic pathways to facilitate tumor growth through angiogenesis, providing oxygen and other nutrients to the tumor [46]. The transcription factor NF-κB modulates caspase and pro-caspase inhibition (primarily caspase 3 and 7), which suppresses central mechanisms of IAP apoptotic pathway [47]. Thus, NF-κB is an essential component of the survivin signaling pathway, which can mediate apoptosis. Therefore, inhibition of NF-κB could render the GBM cells sensitive to chemotherapy; a combination of TMZ and NF-κB inhibitor (Bay 11-7082) may enhance GBM chemosensitivity [47], [48]. Our results suggest this pathway could be modulated to improve therapy outcomes.

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).

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