Journal of Visualized Experiments

A rapid screening workflow to identify potential combination therapy for glioblastoma using patient-derived glioma stem cells

--Manuscript Draft--

Article Type:	Invited Results Article - JoVE Produced Video
Manuscript Number:	JoVE62312R2
Full Title:	A rapid screening workflow to identify potential combination therapy for glioblastoma using patient-derived glioma stem cells
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TITLE:

A Rapid Screening Workflow to Identify Potential Combination Therapy for GBM Using Patient-Derived Glioma Stem Cells

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KEYWORDS:

glioma stem cells, matrigel, drug screening, combination therapy, temozolomide, targeted drug

ABSTRACT:

The glioma stem cells (GSCs) are a small fraction of cancer cells which play essential roles in tumor initiation, angiogenesis, and drug resistance in glioblastoma (GBM), the most prevalent and devastating primary brain tumor. The presence of GSCs makes the GBM very refractory to most of individual targeted agents, so high-throughput screening methods are required to identify potential effective combination therapeutics. The protocol describes a simple workflow to enable rapid screening for potential combination therapy with synergistic interaction. The general steps of this workflow consist of establishing luciferase-tagged GSCs, preparing matrigel coated plates, combination drug screening, analyzing, and validating the results.

INTRODUCTION:

Glioblastoma (GBM) is the most common and aggressive type of primary brain tumor. Currently, the overall survival of GBM patients who received maximal treatment (a combination of surgery, chemotherapy, and radiotherapy) is still shorter than 15 months; so novel and effective therapies for GBM are urgently required.

The presence of glioma stem cells (GSCs) in GBM constitutes a considerable challenge for the

conventional treatment as these stem-like cells play pivot roles in the maintenance of tumor microenvironment, drug resistance, and tumor recurrence[1]. Therefore, targeting GSCs could be a promising strategy for GBM treatment[2]. Nevertheless, a major drawback for the drug efficacy in GBM is its heterogenetic nature, including but not limited to the difference in genetic mutations, mixed subtypes, epigenetic regulation, and tumor microenvironment which makes them very refractory for treatment. After many failed clinical trials, scientists and clinical researchers realized that single-agent targeted therapy is probably incapable of fully controlling the progression of highly heterogeneous cancers such as GBM. Whereas, carefully selected drug combinations have been approved for their effectiveness by synergistically enhancing the effect of each other, thus providing a promising solution for GBM treatment.

Although there are many ways to evaluate the drug-drug interactions of a drug combination, such as the CI (Combination Index), HSA (Highest Single Agent), and Bliss values, etc.[3,4], these calculation methods are usually based on multiple concentration combinations. Indeed, these methods can provide affirmative assessment of drug-drug interaction but can be very laborious if they are applied in high-throughput screening. To simplify the process, a screening workflow for rapidly identifying the potential drug combinations that inhibit the growth of GSCs originated from surgical biopsies of patient GBM was developed. A sensitivity Index (SI) that reflects the difference of the expected combined effect and the observed combined effect was introduced into this method to quantify the synergizing effect of each drug, so the potential candidates can be easily identified by the SI ranking. Meanwhile, this protocol demonstrates an example screen to identify the potential candidate(s) that can synergize the anti-glioma effect with temozolomide, the first-line chemotherapy for GBM treatment, among 20 small molecular inhibitors.

PROTOCOL:

GBM specimen was acquired from a patient during a routine operation after obtaining fully informed consent by human research ethics committee of The First Affiliated Hospital of Nanjing Medical University.

1. Isolation and culture of patient-derived GSCs

1.1. Place fresh surgically resected glioblastoma tissue in a 15 mL centrifuge tube filled with sterile PBS and store the tissue on ice until further operation.

1.2. Mince the GBM tissue into approximately 0.5 to 1 mm diameter pieces using dissection scissors and wash the tissue specimens with neuronal basal medium to remove cellular debris in a biosafety cabinet.

1.3. Digest the tissue fragments with 1 mg/mL collagenase A at 37 °C for 30 min and centrifuge at 400 x *g* for 5 min at 4 °C.

1.4. Remove the supernatant and suspend the pellet with blank neuronal basal medium and dissociate the pellet mechanically by repetitive pipetting on ice.

1.5. Culture the mixture in ultra-low attachment 6-well culture plates filled with GSC culture medium (see **Table 1** for the recipe) in a sterile cell incubator with 5% CO_2 and 90% humidity at 37 °C until neurosphere formation.

1.6. On sufficient neurosphere formation, collect them using a pipette in a 1.5 mL microtube and centrifuge at 800 x *g* for 5 min at room temperature.

1.7. Resuspend the pellet and split it into several flasks filled with the above culture medium for maintaining the primary GSCs.

NOTE: Patient-derived GSCs used in the example were derived from surgical biopsies of a 34-year-old male patient with WHO grade IV recurrent GBM. The GSCs were named as XG387 for the future experiments. PCR-based mycoplasma tests were performed for the above GSCs to confirm no mycoplasma contamination is present. All the experiments involving GSCs used in this protocol were carried out <15 passages.

2. Preparing luciferase-tagged GSCs

2.1. Collect the GSCs from the medium culture and centrifuge them at 70 x *g* for 3 min at room temperature.

2.2. Remove the supernatant, digest the cells with accutase for 4 min at 37 °C. Use a 200 µL tip and pipette repeatedly to dissociate and resuspend the cell pellet.

2.3. Dilute the cells to 2 x 10^5 cells per well in a 12-well culture plate and culture the cells overnight.

2.4. Add 30 µL luciferase-EGFP virus supernatant (titer >10^8 TU /mL) into each well in the plate and then centrifuge the cells at 1,000 x *g* for 2 h at 25 °C. Culture the cells overnight.

2.5. Refresh the medium the next day and culture the cells for another 48 h.

2.6. Observe the cells under a florescent microscope to confirm the appearance of the GFP positive cells.

2.7. Use a flow cell sorter to sort and select the GSCs with high GFP fluorescence to culture the cells further.

3. Bio-luminescence based measurement of cell viability

3.1. Coating plates with the extracellular matrix (ECM) mixture (e.g., Matrigel): Add 40 µL of 0.15 mg/mL ECM mixture to each well and incubate the plate for 1 h at 37 °C. Remove the excess ECM mixture and gently rinse once with PBS.

3.2. Add 100 µL culture medium containing 15,000, 10,000, 8,000, 6,000, 4,000, 2,000, 1,000, and 500 XG387-Luc cells together with 100 µL blank medium as control into each well for 6 replicates in a 96-well optical bottom plate and culture the cells overnight at 37 °C.

3.3. Remove the supernatant, add 50 µL culture medium containing 150 ng/µL D-luciferin into each well and incubate the cells for 5 min at 37 °C.

3.4. Take images of the cellular bio-luminescence in the plate using the IVIS spectrum imaging system. Use the built-in software to create multiple circular areas of the region of interest (ROI) and quantify the cellular bio-luminescence.

4. Temozolomide treatment and combination screening

4.1. Precoat four 96-well plates as described above, prior to the treatment.

4.2. Seed XG387-Luc cells at a density of 1,000 cells in 100 µL culture medium into each well of a 96-well optical bottom plate and culture the cells overnight.

4.3. Prepare temozolomide and the targeted agents from the stock solution in advance. Prepare a concentration series composed of 800 µM, 600 µM, 400 µM, 300 µM, 200 µM, 100 µM, and 50 µM temozolomide in culture medium for the single-agent treatment. Dilute temozolomide and the targeted agents in stock solution in the culture medium, respectively, to obtain final concentrations of 200 µM and 2 µM for combination drug screening (**Table 2**).

4.4. Remove the culture medium when most of the GSCs adhere to the bottom of the plates; add the above-prepared medium containing temozolomide into each well for three technical replicates per treatment.

4.5. To treat Temozolomide and to screen the drug combinations remove the blank medium and add the above-prepared medium containing either 200 µM temozolomide, or 2 µM targeted agent, or a combination of both into each well for three technical replicates per treatment.

4.6. Incubate all plates at 37 °C, 5% CO2 for 3 days.

4.7. Remove the drug-containing medium, add 50 µL blank medium containing 150 ng/µL D-luciferin into each well and incubate the cells for 5 min at 37 °C.

4.8. Take images of the cellular bio-luminescence in the plate using the IVIS spectrum imaging system. Use the built-in software to create multiple circular ROIs and quantify the cellular bio-luminescence.

5. Combination treatment of temozolomide and UMI-77 treatment in XG387-Luc and XG328-Luc cell lines

5.1. Precoat three 96-well plates as described above, prior to the treatment.

5.2. Seed XG387-Luc and XG328-Luc cells at a density of 1,000 cells respectively in 100 µL culture medium into each well of a 96-well optical bottom plate and culture the cells overnight.

5.3. Prepare a concentration series composed of 600 µM, 400 µM, 300 µM, 200 µM, 100 µM, 50 µM, and 0 µM temozolomide and a concentration series composed of 6 µM, 4 µM, 3 µM, 2 µM, 1 µM, 0.5 µM, and 0 µM UMI-77 in the culture medium for six-by-six dose titration matrix treatments.

5.4. Remove the blank medium when most of the GSCs adhere to the bottom of the plate; add the above-prepared medium into each well for three technical replicates per treatment.

5.5. Incubate these plates for 3 days at 37 °C, 5% CO_2.

5.6. Remove the drug-containing medium; add 50 µL of blank medium containing 150 ng/µL D-luciferin into each well and incubate the cells for 5 min at 37 °C for bioluminescence measurement.

6. Data analysis

6.1. Calculate the sensitivity Index (SI) score of temozolomide and targeted agent according to the formula in **Figure 2A**.

NOTE: The SI score is to quantify the influence of the addition of another drug. It ranged from -1 to +1, with positive values indicating temozolomide synergistic effects.

6.2. Calculate the combination index (CI) values between temozolomide and UMI-77 using CompuSyn software to analyze their combined interactions. CI value <1 indicates synergy; CI value >1 indicates antagonism.

6.3. Calculate the high single agent (HSA) values between temozolomide and UMI-77 using Combenefit software. HSA value indicates the combined inhibitory effect. HSA value >0 indicates synergy and the HSA value <0 indicates antagonism.

REPRESENTATIVE RESULTS:

The XG387 cells formed neurospheres in the culture medium described in the **Table 1** in an ultra-low attachment 6-well culture plate or a non-coated plate[5] (**Figure 1A**). First, a test was performed to check whether the bio-luminescence intensity from XG387-Luc cells was proportional to the cell number. As shown in **Figure 1B**, the bio-luminescence intensity increased proportionally to the cell density and resulted in a linear correction between them (Pearson $r = 0.9872$; $p < 0.0001$; **Figure 1C**). Since the bio-luminescence of luciferase tagged cells is easy and quick to measure, this provides a simple method to measure the density of viable GSCs. Next, the anti-proliferative activity of temozolomide was assessed. As shown in **Figure 1D**, 400 µM

temozolomide caused approximately 80% proliferation inhibition of XG387-Luc cells, suggesting it is a useful but its anti-GBM effect can be further improved. The concentration of 200 μM was selected for the combination screening since it is close to the IC50 of temozolomide.

To give an example, 20 target-selective small molecule inhibitors was utilized for the drug combination screening to identify the potential candidate(s) that enhances the anti-GBM effect of temozolomide. As a result, the sensitive index (SI) values of 13 targeted agents were above 0, and 5 of them were above 0.1 (**Figure 2B,C**). Especially, the SI of the top two candidate drugs UMI-77 and A 83-01 were higher than 0.25, suggesting their potential to synergize with temozolomide.

To validate the above finding, the classical synergy models of HSA and Bliss[3,4,6] were applied to determine the combined effect of temozolomide and UMI-77 in GSCs. In addition, XG328—another patient-derived GSC model established early—was used to perform the same evaluation. Anti-proliferative assay of the combined treatment of temozolomide and UMI-77 was performed in a six-by-six dose titration matrix. The results were analyzed to acquire the HSA and Bliss values which are readouts for synergistic inhibition and depict the difference between the expected inhibition and the observed inhibition. As shown in **Figure 3B** and **Figure 3C**, the combination index (CI) values <1 and the high single agent (HSA) values >0 for most of the combinations of temozolomide and UMI-77 at different concentrations, suggests an overall synergistic interaction of temozolomide and UMI-77 in both XG387 and XG328 GSCs.

FIGURE LEGENDS:

Figure 1: GBM patient-derived GSCs XG387. (**A**) Neurosphere formation. (**B**) Bio-luminescence generation of luciferase tagged GSCs. (**C**) Bio-luminescence generated by XG387-Luc cells was proportional to the cell density. (**D**) Temozolomide treatment of XG387. Each treatment was performed in triplicate with two independent experiments. The data are expressed as the mean ± SD.

Figure 2: Drug combination screening using GSCs. (**A**) The formula to calculate the SI (sensitivity index) of 20 targeted agents with temozolomide (TMZ) in the combination screen. (**B**) The distribution of SI values of 20 targeted agents. Red dots: the top five candidate drugs. (**C**) Information of the top five candidate targeted agents.

Figure 3: Combination treatment of temozolomide (TMZ) and UMI-77 in XG387-Luc and XG328-Luc cell lines. (**A**) Single and combinatorial titration of temozolomide and UMI-77 in a proliferation assay in XG387-Luc and XG328-Luc cell lines. (**B**) Isobologram and combination index analysis of the proliferation inhibition in XG387-Luc and XG328-Luc cells treated with temozolomide and UMI-77. CI <1 indicates a synergistic effect. (**C,D**) Synergy plots generated by Combenefit showing the interaction between temozolomide and UMI-77. Analysis of interaction resulted in HSA (high single agent) values and Bliss values, indicating synergistic efficacy as calculated from the expected. HSA and Bliss values >0 indicate synergistic effects. Each treatment was performed in triplicate with two independent experiments.

DISCUSSION:

In the present study, a protocol that can be applied to identify potential combination therapy for GBM using patient-derived GSCs was described. Unlike the standard synergy/additivity metric model such as Loewe, BLISS, or HSA methods, a simple and quick workflow was used that does not require a drug pair to be combined at multiple concentrations in a full factorial manner as the traditional methods. In this workflow, SI (sensitivity index) which is originated from a study to evaluate the sensitizing effect of siRNAs in combination with small molecular inhibitor was introduced to quantify the synergistic drug effect of two small molecular inhibitors[7]. The range of SI values is from -1 to 1, and the positive SI value indicates a sensitizing effect between each of the drugs. The higher the SI value achieved, the stronger the synergy was. Although the SI value alone is incapable to provide an affirmative answer about the type (synergistic, additive, or antagonistic) of drug-drug interaction, those top-ranked candidates have high probability to synergize with the drug of interest and therefore are worthy of further validation. In comparison, most of the current high-throughput drug combination screening methodologies are still laborious and involve difficult algorithms[8,9].

To exemplify the feasibility of this method, a small-scale test screen was performed. As a result, it was possible to identify UMI-77, a selective MCL1 inhibitor, as the top candidate among 20 targeted agents to synergize with temozolomide in GSCs growth suppression. In fact, in a previous study, UMI-77 was also found to synergistically enhance the anti-glioma activity of temozolomide in established GBM cells[10]. In the current study, the synergistic interaction between UMI-77 and temozolomide was approved again in GSCs using the classical Chou-Talalay combination index, BLISS or HSA methods. Another advantage of this protocol is the usage of luciferase-tagged GSCs for measuring the viable proportion of cells. The luciferase activity of cells can be easily measured by the addition of the luciferin, the substrate of luciferase, and capture the luminescence by any instrument with the function of luminometric measurement. Because the luciferase-luciferin reaction is quick, herein it provides a cheap and quick solution in comparison with traditional MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium), or CCK-8 (cell counting kit-8) assays, all of which require long incubation times. Together, the protocol presents a high-throughput screening of potential drug combination for GBM. The protocol also provides optional quick and simple solution for drug combination screen in addition to the standard synergy evaluation methods.

DISCLOSURES:

The authors declare no conflicts to disclose.

ACKNOWLEDGMENTS:

We thank The National Natural Science Foundation of China (81672962), the Jiangsu Provincial Innovation Team Program Foundation, and the Joint Key Project Foundation of Southeast University and Nanjing Medical University for their support.

REFERENCES:

1. Lathia, J. D., Mack, S. C., Mulkearns-Hubert, E. E., Valentim, C. L., Rich, J. N. Cancer stem cells

in glioblastoma. *Genes & Development*. **29** (12), 1203–1217 (2015).
2. Binello, E., Germano, I. M. Targeting glioma stem cells: a novel framework for brain tumors. *Cancer Science*. **102** (11), 1958–1966 (2011).
3. Mathews Griner, L. A. et al. High-throughput combinatorial screening identifies drugs that cooperate with ibrutinib to kill activated B-cell-like diffuse large B-cell lymphoma cells. *Proceedings of the National Academy of Sciences of the United States of America*. **111** (6), 2349–2354 (2014).
4. Di Veroli G. Y. et al. Combenefit: an interactive platform for the analysis and visualization of drug combinations. *Bioinformatics*. **32** (18), 2866–2868 (2016).
5. Shi, Y. et al. Ibrutinib inactivates BMX-STAT3 in glioma stem cells to impair malignant growth and radioresistance. *Science Translational Medicine*. **10** (443), 1–13 (2018).
6. Tan, X. et al. Systematic identification of synergistic drug pairs targeting HIV. *Nature Biotechnology*. **30** (11), 1125–1130 (2012).
7. Jansen, V. M. et al. Kinome-wide RNA interference screen reveals a role for PDK1 in acquired resistance to CDK4/6 inhibition in ER-positive breast cancer. *Cancer Research*. **77** (9), 2488–2499 (2017).
8. Malyutina, A. et al. Drug combination sensitivity scoring facilitates the discovery of synergistic and efficacious drug combinations in cancer. *PLoS Computational Biology*. **15** (5), e1006752 (2019).
9. He, L. et al. Methods for High-throughput drug combination screening and synergy scoring. *Cancer Systems Biology*. **1711**, 351–398 (2018).
10. Chen, C. et al. Targeting the synthetic vulnerability of PTEN-deficient glioblastoma cells with MCL1 inhibitors. *Molecular Cancer Therapeutics*. **19** (10), 2001–2011 (2020).

Figure 1

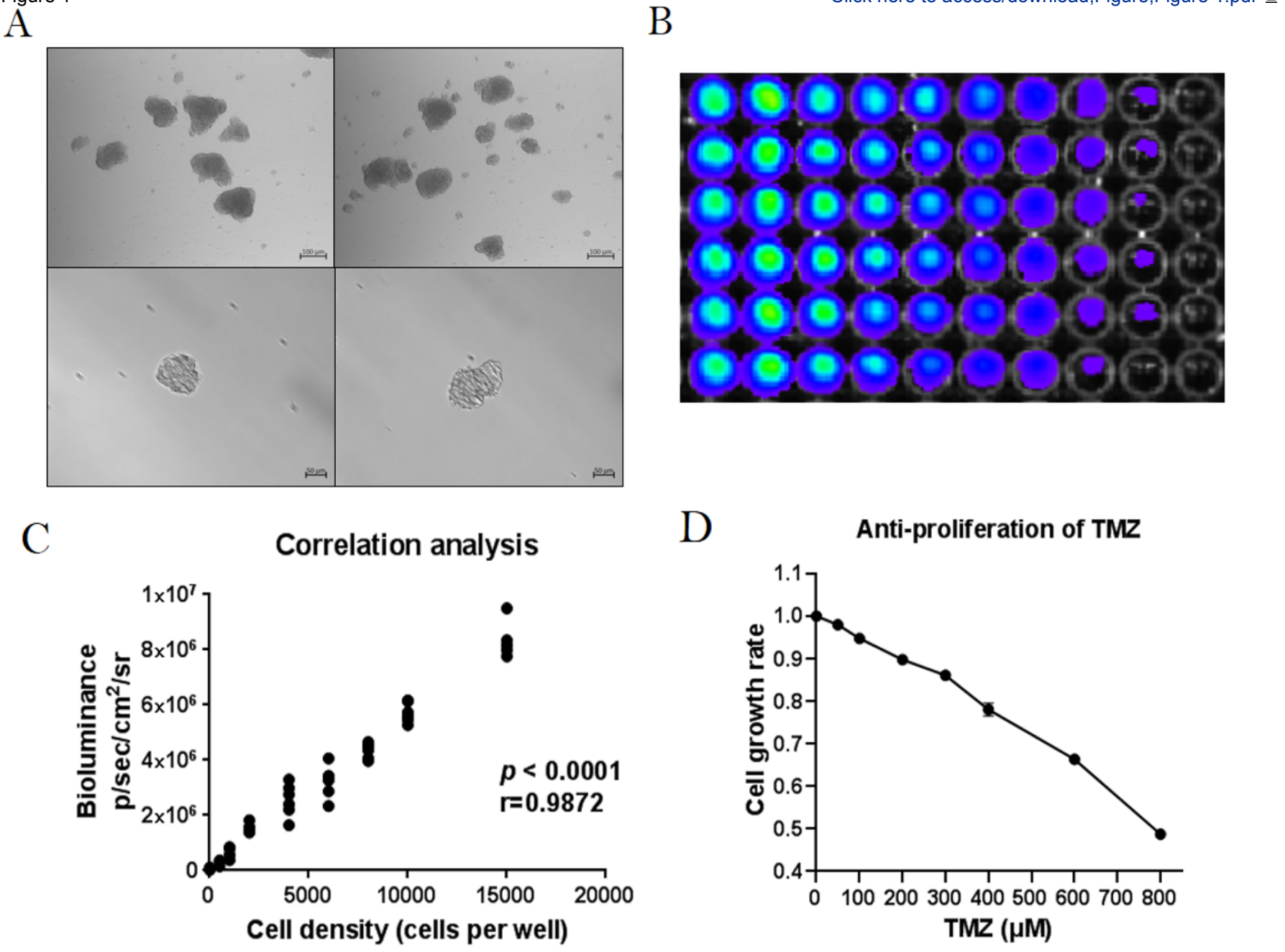


Figure 2

A

Formula to calculate the SI (Sensitivity Index)

p1: The proliferation rate of GSCs treated with targeted agent
cc: The proliferation rate of GSCs treated with vehicle
cp: the proliferation rate of GSCs treated with combination
p2: the proliferation rate of GSCs treated with temozolomide

Effect of targeted agent p1/cc

Effect of TMZ p2/cc

Expected combined effect: EE

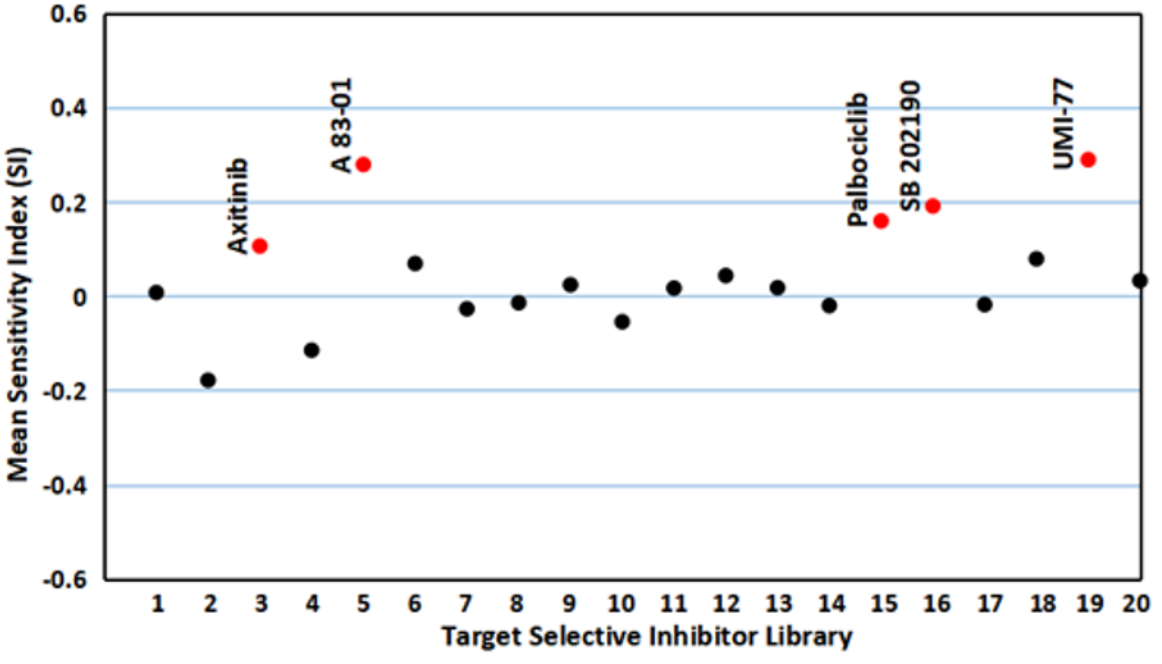
p1/cc × p2/cc

Observed combined effect: OE

cp/cc

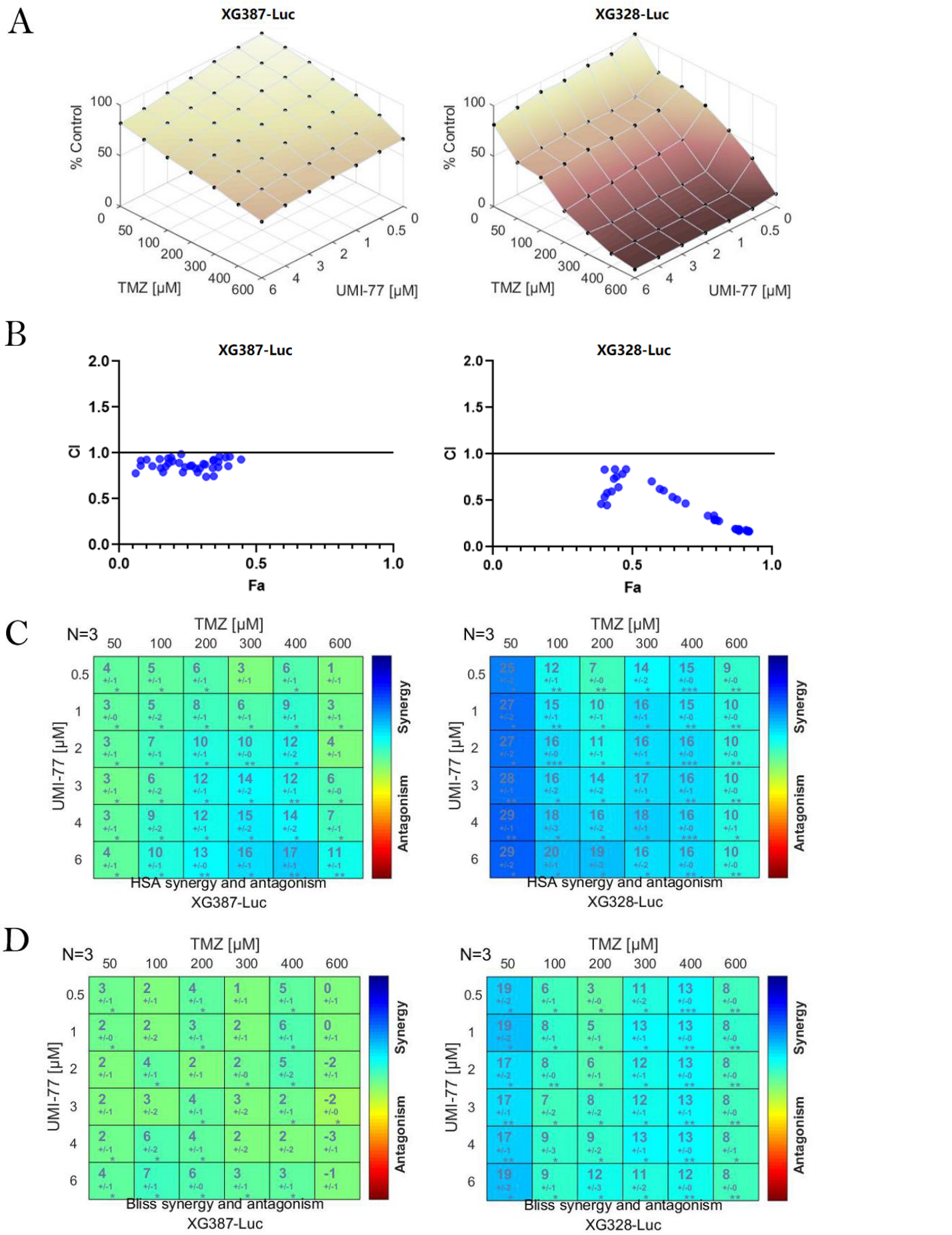
SI = EE – OE > 0: synergistic
Range: -1 to 1 <0: antagonistic

B



C

	Targeted agent	SI value	Target(s)	Pathway
1	UMI-77	0.28972	Mcl-1	Apoptosis
2	A 83-01	0.27939	ALK5, ALK4, ALK7	Others
3	SB 202190	0.19123	p38α, p38β2	MAPK-p38
4	Palbociclib	0.15962	CDK4, CDK6	Cell cycle
5	Axitinib	0.10635	VEGFR1-3, PDGFRβ	tyrosine kinase (RTK) signaling pathway



Name of Material/Equipment	Company	Catalog Number	Comments/Description
B-27	Gibco	17504-044	50X
EGF	Gibco	PHG0313	20 ng/ml
FGF	Gibco	PHG0263	20 ng/ml
Gluta Max	Gibco	35050061	100X
Neurobasal	Gibco	21103049	1X
Penicillin-Streptomycin	HyClone	SV30010	P: 10,000 units/ml S: 10,000 ug/ml
Sodium Pyruvate	Gibco	2088876	100 mM

Table 1. The formulation of GSC complete culture medium.

Number	Name of Material/Equipment	Company	Comments/Description
1	ABT-737	MCE	Selective and BH3 mimetic Bcl-2, Bcl-xL and Bcl-w inhibitor
2	Adavosertib (MK-1775)	MCE	Wee1 inhibitor
3	Axitinib	MCE	Multi-targeted tyrosine kinase inhibitor
4	AZD5991	MCE	Mcl-1 inhibitor
5	A 83-01	MCE	Potent inhibitor of TGF-β type I receptor ALK5 kinase
6	CGP57380	Selleck	Potent MNK1 inhibitor
7	Dactolisib (BEZ235)	Selleck	Dual ATP-competitive PI3K and mTOR inhibitor
8	Dasatinib	MCE	Dual Bcr-Abl and Src family tyrosine kinase inhibitor
9	Erlotinib	MCE	EGFR tyrosine kinase inhibitor
10	Gefitinib	MCE	EGFR tyrosine kinase inhibitor
11	Linifanib	MCE	Multi-target inhibitor of VEGFR and PDGFR family

12	Masitinib	MCE	Inhibitor of c-Kit
13	ML141	Selleck	Non-competitive inhibitor of Cdc42 GTPase
14	OSI-930	MCE	Multi-target inhibitor of Kit, KDR and CSF-1R
15	Palbociclib	MCE	Selective CDK4 and CDK6 inhibitor
16	SB 202190	MCE	Selective p38 MAP kinase inhibitor
17	Sepantronium bromide (YM-155)	MCE	Survivin inhibitor
18	TCS 359	Selleck	Potent FLT3 inhibitor
19	UMI-77	MCE	Selective Mcl-1 inhibitor
20	4-Hydroxytamoxifen(Afimoxifene)	Selleck	Selective estrogen receptor (ER) modulator

Table 2. The information of 20 targeted agents used in the test screen. All of these are target selective small molecular inhibitors. The provider, name, and targets were given in the table.

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[mailto:em.jove.24f40.704c5a.e10e80fe@editorialmanager.com] **On Behalf Of** Vidhya Iyer
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Subject: Revisions required for your JoVE submission JoVE62312 - [EMID:c1ef672158d5805b]

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13. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Answer: Done.

14. Please remove the titles and Figure Legends from the uploaded figures and move them to a Figure Legends section after the Representative Results.

Answer: The Figure legends were moved to the manuscript below the representative results.

15. Please ensure that the corresponding reference numbers appear as numbered superscripts after the appropriate statement(s) for in-text formatting.

Answer: The citation style was changed to numbered superscripts.

16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Answer: The format of references have been corrected.

17. Figure 1: Please include scale bars in all the figures of the panel and include the details in the Figure Legends.

Answer: The scale bars were included in the corresponding figures.

18. Please sort the Table of Materials in alphabetical order.

Answer: Relevant changes have been made in the Table of Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The objective of the manuscript was to establish a quick, straightforward experimental approach to identify synergistic drug interactions between the alkylating agent temozolomide and clinically actionable drug targets. The authors show efficacy in cell isolation from a clinical Glioblastoma specimen. Bioluminescence is employed as a reporter for cell viability, within a dynamic range for quantitative and qualitative analysis. From the 20 small molecule inhibitors, 65% demonstrated synergy in cell viability with an IC50 dose of temozolomide. The authors describe a statistical method for calculating drug synergy based on the Loewe additivity model. While conducting drug synergy analysis on a single concentration combination certainly simplifies preclinical drug studies, this procedure diminishes scientific rigor and reproducibility. Even though technical replicates improve the validity of this procedure, biological replicates of varying concentrations are necessary to analyze the true scope of variance of combination therapies. With this said, this manuscript will benefit from substantial revisions in study design.

Major Concerns:

Lines 130-135 : "Especially, the SI of the top two candidate drugs UMI-77 and A 83-01 were higher than 0.25, suggesting their potential to synergize the anti-GBM effect of temozolomide is promising". To substantiate this statement additional patient derived models are required.

Answer: To confirm that this is not a XG387 GSCs dependent effect, we took advantage of another patient derived GSC model established early in our lab and further tested the drug-drug interaction of UMI-77 and temozolomide on it.

Lines 146-148: "To simplify the process, we used a screening workflow for rapidly identifying potential drug combinations that inhibit the growth of GSCs originated from surgical biopsies of patient glioblastoma". As this analytical method is central to the manuscript, the performance of this methods needs to be tested for specificity and sensitivity alongside a gold standard methodology (such as Bliss).

Answer: We applied the Bliss and HSA models to confirm the finding that UMI-77 synergized the GSCs inhibitory effect of temozolomide. In addition, in a previous study, we reported that temozolomide and UMI-77 have synergistic anti-glioma effect in established GBM cell lines (Mol Cancer Ther. 2020;19(10):2001-11). These results/information were all included in the manuscript.

Figure 1A: missing scale for phase contrast microscopic images

Answer: The scale bars were added in the corresponding figures.

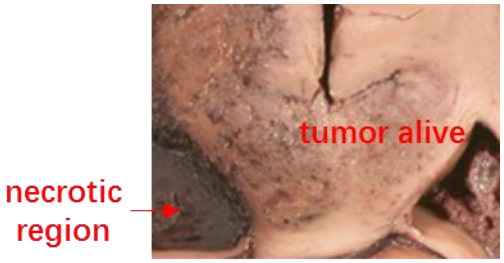
Figure 1C: could show correlation analysis for cell density and luminescenc

Answer： The correlation analysis was shown in Fig. 1C.

Figure 1C, D: number of technical replicates is not mentioned.

Answer: All experiments except the drug screen were performed in triplicate with two independent experiments. This is also mentioned in the manuscript.

Minor Concerns:
"Those pieces containing substantial amounts of necrosis were removed". Please elaborate on how necrosis was identified and what were the steps that were employed to separate necrotic areas.
Answer: According to experience, the necrotic region in the tumor appears to be the black or dark red solid mass that caused by blockage of intratumor vessels and blood clotting inside of the tumor.



"quantified by Living Image 4.2". More information is needed in regards to specifying ROI (region of interest) and data acquisition

Answer: We quantified the intensity of photons of each well in the 96-well plate by creating multiple circle area of ROI using Living Image 4.2 built-in function.

Line 104-106: "We previously established a formula to calculate the high-drug combination screening in which a Sensitivity Index (SI) score was introduced to quantify the influence of the addition of another drug (1)". Check the reference as this appears to reference the 2020 Mol Can Ther manuscript.

Answer: We have removed this part of text including this citation.

Numerous grammatical errors noted:

i.e. Abstract: "The presence of GSCs making the GBM very refractory to most of

individual targeted agents"
Answer: “making” was replaced by “makes”.

Lines 114-115: "A series of the density of XG387-Luc cells"

Answer: Corrected to “A series of densities of XG387-Luc cells”.

Lines 135-137: "The heterogenetic nature of GBM, such as the difference in genetic mutations, subtypes, epigenetic regulation, and tumor microenvironment, making them refractory cancers for targeted therapy
Answer: “making” was replaced by “which makes”.

Reviewer #2:
Manuscript Summary:
This manuscript describes a protocol for screening GSCs to identify potential drug combinations for glioblastoma therapy. The methodology includes isolating and culturing presumed GSCs from GBM tumor tissue, establishing luciferase-tagged GSC cells, combination drug screening, and analysis of resulting data. The authors tested the ability of 20 small molecule inhibitors to synergize with the standard of care drug, temozolomide (TMZ), in one patient-derived GSC line. Based on Sensitive Index (SI) values the authors identify lead candidates such as UMI-77 and A 83-01 as having the potential to synergize with TMZ.

Major Concerns:
This protocol is of interest to researchers working on glioblastoma as well as other cancer researchers. However, a major concern is that only one patient-derived GBM cell line has been utilized. The efficacy and reproducibility of the protocol can not be clearly demonstrated with one sample. Have other GBM patient-derived cells been tested? The authors also do not provide any methodology or data on how they validated the stem like properties of the cells used for screening. Were any experiments performed to confirm the stem cell phenotype? The Discussion does not adequately address how the authors method and formula to calculate SI compares to other HTS screening protocols and statistical models to evaluate drug combination efficacy.

Minor Concerns:
No passage numbers are provided for the cells. Were the cells tested for mycoplasma prior to use in experiments?

Answer: We confirmed that all GSCs are mycoplasma negative by PCR-based mycoplasma test before formal experiments. All experiment involved GSCs were carried out <15 passages.

This should be stated in the manuscript.
Line 125: "closed" should be replaced with "close".

Answer: Corrected.

Line 140: "control" should be "controlling".

Answer: Corrected.

There is no scale bar for Figure 1A.

Answer: The scale bars were included in the corresponding figures.

Reviewer #3:
Most of the suggestions I have are based on punctuation/grammar. I see no major issues with the methods presented here. However, there are a few details that could be added to make it easier for the reader to replicate the technique. Additionally, I highly recommend modifying the recipe of your GSC medium for future experiments if possible (as explained below).

Punctuation/Grammar:

Abstract:
The glioma stem cells (GSCs) are a small fraction of cancer cells which play essential roles in tumor initiation, angiogenesis, and drug resistance in glioblastoma (GBM), the most prevalent and devastating primary brain tumor. The presence of GSCs makes the GBM very refractory to most of individual targeted agents, so high-throughput screening methods are required to identify potential effective combination therapeutics. Here, we describe a simple workflow to enable rapid screening for potential combination therapy with synergistic interaction. The general steps of this workflow consist of establishing luciferase-tagged GSC cells, preparing matrigel coated plates, combination drug screening, analyzing and validating the results.

Answer: We are thankful for the kind helps on punctuations/grammars. The corresponding corrections have been made in the manuscript.

Line 47:
plates (Corning, NY, USA) filled with GSC culture medium (see Table 1 for the recipe) in a cell

Line 48:
incubator within a 37 ° C, 5% CO2, and 90% humidity sterile incubator until formation of

Line 51:
resuspended and split into several flasks filled with the above culture medium for maintaining

Line 65:
2.4 GSCs with high GFP fluorescence were selected for further culturing by cell sorting using a BD

Line 69:
To validate that the bio-luminescence of the luciferase tagged cells could be used to

Line 71/72:
3.1 100 μl culture medium containing 15000, 10000, 8000, 6000, 4000, 2000, 1000, and 500 XG387-Luc cells, together with 100 μl blank medium as control, were added into each well for six

Line 74:
3.2 In The next day, after removal of the supernatant, 50 μl culture medium containing 150 ng/μl

Line 92:
containing temozolomide was added into each well for three technical replicates per treatment. No

Line 93:
medium refreshment was required until bio-luminescence measurement.

Line 96:
agent, or a combination of both was added into each well for three technical replicates per treatment.

Line 97:
No medium refreshment was required until bio-luminescence measurement.

Line 112:
The XG387 formed neurospheres in the culture medium described in the Table 1 in an

Line 114:
the bio-luminescence intensity from GSC-Luc cells was proportional to the cell number. A series

Line 115:
of the density of XG387-Luc cells were seeded in a 96-well plate with a clear bottom, and their

Line 116:

luciferase activities were measured by bio-luminescence generated per unit time after addition of

Line 126:
IC50 of temozolomide.

Line 128:
inhibitors with specific targets in an attempt to screen for a potential candidate that enhances the anti-

Line 136:
epigenetic regulation, and tumor microenvironment, makes them refractory cancers for

Line 140/141:
incapable of efficiently controlling the progress of highly heterogeneous cancers like GBM. However, carefully selected drug combinations can synergistically enhance the efficacy of each other,

Line 161/162:
quick solution relative to the traditional methods that depend on MTT, MTS, or CCK-8 agents,
162 all of which tend to require long incubation times. In addition, the luciferase tagged GSCs can also

Figure 1:
Figure 1. Glioblastoma patient-derived GSCs XG387. (A) Neurosphere formation; (B) Bio-luminescence generation of luciferase tagged GSCs; (C) Bio-luminescence generated by XG387-Luc cells was proportional to the cell density; and (D) Temozolomide treatment of XG387.

Answer: We have reviewed the above suggestions and made corresponding corrections.

Technical Suggestions:

On line 54, the authors cite the virus used, but I think it would be helpful to include the titer/MOI as the subsequent info only mentions the total volume used (30 uL).

Answer: The Luciferase-EGFP virus is a commercial lenti-viral stock. The titer was higher than 10^8 TU /ml according to the supplier.

On line 80, it would be useful to include the concentration of the Matrigel in addition to the total volume used.

Answer: This concentration of Matrigel (0.15 mg/mL) was given in the manuscript.

In the future, I would suggest to use Neurobasal-A medium (without Vitamin A) instead of Neurobasal medium because the presence of vitamin A can decrease self-renewal and cause differentiation. Although the relatively small amount of vitamin A in your media likely did not cause a significant effect on these experiments, it could be a problem if you were analyzing more sensitive effects, such as changes in RNA sequencing or other dynamic epigenetic changes.

Answer: Thanks for the very helpful information.

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24 Nov 2020

Dear Editor of JOVE:

I was very glad to receive your invitation to contribute to your special collection of "Overcoming Therapeutic Barriers in Glioblastoma". And now on behalf of all authors, I am pleased to submit a manuscript titled "A rapid screening workflow to identify potential combination therapy for glioblastoma using patient-derived glioma stem cells" to JOVE for consideration.

In this manuscript, we described a protocol that began with isolation and culture the primary glioma stem cells from a glioblastoma patient, till they formed neuro-spheres and were infected with luciferase, and finally were used for a drug combination screening. Moreover, we proposed a simple formula to rapidly identify the potential drug combination and applied it for a test screen in which those targeted agents to enhance the anti-glioma effect of temozolomide were identified. We hope this manuscript will fit the scope of this collection and meanwhile the interest to the readership of JOVE.

We confirm that the work presented in this manuscript has not been published elsewhere, nor is it currently under consideration by another journal. It has been read and approved by all the co-authors and there is no financial or other conflicts of interest.

We appreciate your efforts in reviewing this manuscript.

Yours sincerely,

Lin Fan