Inhibiting stemness and invasion property of glioblastoma tumorsphere by a newly designed biguanide

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Inhibiting stemness and invasion property of glioblastoma tumorsphere by a newly designed biguanide

Directed by Professor Se Hoon Kim

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Junjeong Choi

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ABSTRACT

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Glioblastoma (GBM) is a lethal disease with limited survival in spite of recent therapeutic progress and a new strategy against the disease is being required. Recently, the effect of biguanide derived agents are being suggested as new groups of target in the treatment of malignant tumors and their effect against the tumorsphere (TS) were reported. In this study, I assessed effects of a newly developed biguanide, HL156A, on the properties of glioblastoma tumorsphere (GBM-TS) and survival of orthotopic xenograft animals, to assess the feasibility of this agent, alone or combined with conventional therapeutic agent temozolomide (TMZ), in the treatment of GBM. HL156A, alone and

combined with TMZ, exhibited an inhibitory effect on the stemness of GBM-TS, proven by the neurosphere formation assay and the assessment of stemness marker expression, without affecting viability of cells. The invasive property of GBM-TS were inhibited most significantly by the combination treatment, compared with the control and HL156A or TMZ alone-treated groups in 3-dimentional collagen matrix invasion assay. The combination treatment repressed epithelial-mesenchymal transition (EMT) related gene expression. Gene ontology class comparison of TMZ and combination treatment group revealed altered expression of gene sets involving cellular adhesion and migration. The combined treatment of HL156A and TMZ showed survival benefits in the orthotopic xenograft mouse model. Targeting of GBM-TSs by the combination of HL156A and TMZ, through the inhibition of stemness and invasion properties of GBM- TS, can be a novel candidate for the treatment of GBM.

Key words: biguanide, glioblastoma, HL156A, invasion, stemness, tumorsphere

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I. INTRODUCTION

1. Glioblastoma

Glioblastoma (GBM) is the most fatal brain tumor, showing a very limited survival rate in spite of best treatment. Standard first line therapy is a surgery followed by concurrent temozolomide (TMZ) treatment and radiation. According to National Comprehensive Cancer Network (NCCN) guidelines of central nervous system cancer, only a third of patients survive for one year and less than 5% lives beyond 5 years.[1] Temozolomide, an alkylating (methylating) agent, is now the standard of care in conjunction with postoperative radiation therapy (RT) for younger, good performance patients with GBM.[1] Stupp et al

conducted a phase III, randomized study that assessed the drug in 573 GBM patients' age ≤70 years with a WHO PS(performance status) of 2 or less. In this study, the chemoradiation group resulted in a statistically improved median survival (14.6 vs. 12.1 months) and 2-year survival rate (26.5% vs. 10.4%) compared with radiation alone.[2] However, the patients ultimately succumb to death by the relapse of the disease.

Understanding of molecular genetics of GBM was accelerated by the finding that O^6-methylguanine–DNA methyltransferase (MGMT) methylation could be a good predictor of tumor treatment by alkylating agent, TMZ.[3] The monumental project, the cancer genome atlas (TCGA) study even more provided tremendous information of genome of GBM. Using high-throughput studies and bioinformatics analysis, it was shown that there exist four molecular subtypes of the tumor: classical, mesenchymal, proneuronal and neural types. In addition, they showed response to the aggressive therapy differed by subtype, with the greatest benefit in the classical subtype and no benefit in the proneural subtype.[4] Further study revealed the existence of a glioma-CpG island methylator phenotype (G-CIMP) with characteristic clinical phenotypes and survival advantage of the proneural subtype is conferred by the G-CIMP

phenotype, and MGMT DNA methylation may be a predictive biomarker for treatment response only in classical subtype GBM.[5-6]

2. Cancer stem cell hypothesis

Growing body of evidence that supports the idea that cancers are initiated and maintained by a subpopulation of cells are being reported. Ever since the compelling evidence proving existence of cancer stem cells (CSCs) in acute myeloid leukemia by Bonnet and Dick in 1997, CSCs have been identified in multiple human malignancies including breast, brain, colon, ovary and pancreas.[7-12] The ideas that the organization of cell lineage in tumors is hierarchical and only a subset of cells is responsible for tumor progression are main subjects of this theory. Adult stem cells and their progenitors are being recognized as one of sources of tumor initiating cells. Regardless of the origin, CSC is defined by its stem cell-like properties like self-renewing capability, generating a hierarchy organization harboring varied downstream descent, proliferating extensively and initiating tumor.[13] The presence of CSCs raises the clinical implication that a curative therapy will require complete elimination of this unique population in patients with an initial response to treatment, since

the disease may ultimately recur if even a small number of CSCs survive the therapy. Interestingly, accumulated evidence has established that CSC populations are more resistance to conventional cancer therapy than non-CSC population. For example, CD133 positive GBM CSCs displayed strong capability on tumor's resistance to chemotherapy and radiotherapy.[14,15] Consequently, novel therapeutic systems have been developed with the purpose of targeting CSCs and altering the microenvironment these cell potentially resides. Targeting surface of markers, signaling cascades, microenvironments were being tried and examined.[16]

Recent experimental data suggest that CSCs are responsible for GBM invasiveness, a most important characteristic of the tumor that renders complete tumor resection almost impossible. Cells enriched for the putative stem cell marker C133 display greater migratory and invasive potential *in vitro* and *in vivo* when compare with matched CD133-negative tumor cells derived human primary GBMs, GBM xenograft and brain tumor cell lines.[14,17] Moreover, putative stem cell marker-positive cells were found in the leading edge of the tumor.[18] Given that there is a notion that CSCs are functionally heterogeneous in terms of surface marker expression, neurosphere formation ability, and *in*

vivo tumorigenic potential, it is possible that CSCs switch from a stationary and proliferative phenotype to a migratory one and *vice versa*, ensuring the enlargement of the tumor core and the colonization of the neighboring normal brain tissue.[19,20]

3. Epithelial to mesenchymal transition in glioblastoma

Although originally identified as a process in the development, epithelial to mesenchymal transition (EMT) is a process that allows a differentiated epithelial cells, entirely settled and patterned to establish stable contacts with neighbor cells, to acquire a mesenchymal cell phenotype, characterized by loss of cell to cell interactions,[21] reduced cellular adhesion,[22] increased cytoskeletal dynamic[23] and changes in transcription factor,[24] all of which ultimately lead to increased migration and invasion property. Adapting this unique process by cancer cells was known to be associated with the acquisition of a stem cell program.[25] SNAI1, TWIST and Zinc-finger enhancer binding (ZEB) family members regulate the program and the activity of them has been reported to be altered in GBM. SNAI is known to be involved in the regulation of glioma cell proliferation and migration.[19,26] The ZEB2 expression

level was significantly increased in glioma and the silencing of the molecule reduced the expression of mesenchymal cell markers.[27] Mesenchymal subtype of GBM was identified in TCGA data set and high expression of genes associated with mesenchyme derived tissue is related with poor overall survival and treatment resistance.[4] They speculated this could be considered reminiscent of the EMT program.[4] Another speculation is that aberrant activation of EMT factors during tumorigenesis can trigger the mesenchymal shift in GBM.[28]

4. Biguanide as a potential therapeutic agent for the treatment of GBM through targeting of cancer stem cells

Since studies unveiled that the biguanide derivate, metformin (N',N'-dimethylbiguanide), the most widely used oral therapeutic agent to lower blood glucose concentration in patient with type 2 diabetes, significantly reduced cancer incidence and improved cancer pateints' survival in type 2 diabetics, laboratory evidence of antineoplastic effect of biguanide has been accumulated and first generation of clinical trials on metformin in progress are anticipated.[29,30] Direct action of biguanides on transformed cells or cells at risk for transformation was attributed to a consequence of homeostatic response to

the agent-induced energetic stress or may be attributed to energy depletion by the inhibition of oxidative phosphorylation (OXPHOS) which leads to the energy conservation state or 5' adenosine monophosphate-activated protein kinase(AMPK) activation within the cells, but still under investigation. Remarkably, Hirsch et al. have demonstrated that mass-forming, self-renewing tumor initiating breast cancer cells seems to exhibit an exacerbated sensitivity to metformin.[31] This group suggest that TGF-β-induced EMT might represent a common molecular mechanism underlying the anti-cancer stem cells actions of metformin.[29] However, supporting studies for this notion are still limited.

In the present study, I assessed the effect of HL156A, alone or combined with conventional therapeutic agent, TMZ, on the properties of GBM-TS and survival of orthotopic xenografted animals, to evaluate the feasibility of new drug, as a potential therapeutic agent for GBM.

II. MATERIALS AND METHODS

1. Cells

GSC11, established at M.D. Anderson Cancer Center-Houston from primary gliomas removed from glioma patients and the X01 line, derived from a woman with GBM were used for experiment.[32,33]

2. Lentiviral vector transduction and expression

GFP stably expressed GSC11s (G-GSC11) were generated by growing GSC11 cells in complete medium and then applying GFP-expressing lentiviral supernatants. Polybrene (Sigma) was added to a final concentration of 8 μg/ml and incubated with cells for 18 hours. After infection, the cells were placed in fresh growth medium and cultured in a standard manner. Cells were treated with 1 mg/ml puromycin (Life Technologies Korea, Seoul, Korea) to eliminate uninfected cells and generate stable G-GSC11. G-GSC11s were isolated for use in further experiments by fluorescence‐activated cell sorting (FACS).

3. Cell viability assay

The effect of HL156A, TMZ and combined HL156A and TMZ on the survival of cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates and incubated at 37°C for 24 hours and treated with HL156A, TMZ and their combination for 5 days. MTT reagent (10μl/well) was added, incubated at 37°C for 4 hours and the absorbance was measured at 490nm. Each experiment was repeated three times in triplicate and the results were expressed as % viable cells over control.

4. Western blotting

Total protein of 20 μg from each sample was treated with Laemmli sample buffer and heated at 100℃ for five minutes. Then it was loaded into each well and was resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes (GE Healthcare life-Sciences). Membranes were blocked in 5% non-fat dry milk in TBS-T, and incubated with antibodies for AMPK-mammalian target of rapamycin (mTOR) pathway related proteins, stemness markers, EMT-related markers, overnight at

4°C, and then probed with peroxidase-conjugated goat anti-rabbit IgG (1:2000, Santa Cruz Biotechnology INC., CA, USA) for 1 hour at room temperature. The washes were repeated and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). Band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

5. Neurosphere formation assay

The GSC11 and X01 cells were cultured in medium consisting of DMEM/F-12 with 2 % 1×B27, 20 ng/ml 0.02 % bFGF, 20 ng/ml of 0.02 % EGF, and 1 % antibiotic–antimycotic solution (100×, Gibco, Invitrogen Korea, Seoul, Republic of Korea). The cells were cultured in different conditions for 3 weeks. Cell cultures were observed with an inverted phase-contrast microscope (I×71 Inverted Microscope; Olympus, Tokyo, Japan) to determine morphology and the size of the tumor sphere. Photographs of cells were obtained with a digital camera (DP70 Digital Microscope Camera; Olympus), using DP Controller software (Olympus, Tokyo, Japan).

6. Three dimensional invasion assay

The G-GSC11s grown in spheroid i.e. tumorsphere (TS) were cultured

in collagen I matrices using polydimethylsiloxane (PDMS)-based micro-wells (diameter and depth of microwells: 6mm and 500μm). The microwells were treated with 1% poly (ethyleneimine) (Sigma-Aldrich, St. Louis, MO, USA) solution for 10 minutes followed by 0.1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 30 mins and washed with PBS for overnight to make PDMS wells adherent to collagen. The 4mg/ml collagen I matrices are prepared from high-concentration rat tail collagen I (BD Bioscience, CA, USA) using the recommended manual provided by the manufacturer. Briefly, the amounts of 10x phosphate buffered saline (PBS), 1N NaOH, sterile dH2O and collagen I were mixed to create gels based on the desired final concentration. The solution is well mixed and kept at 4℃ before use. To encapsulate G-GSC11 spheroid, 10ul of collagen I solution (4mg/ml) were pipetted into the micro-well, single G-GSC11 TS is placed onto collagen I matrices from culture plate, and 10ul of collagen I solution (4mg/ml) was dropped onto G-GSC11 TS. The platform was incubated at 37℃ and 5% CO2 for 30 mins. The cell viability was characterized by staining G-GSC11 TS with 8μM Ethidium homodimer-1 (Invitrogen Korea, Seoul, South Korea) for 30 mins at 37℃ before implantation

in collagen matrix. After full gelation, a superlayer of culture medium consisting of DMEM/F-12 with 2 % 1×B27, 20 ng/ml 0.02 % bFGF, 20 ng/ml of 0.02% EGF, and 1% antibiotic–antimycotic solution (100×, Gibco, Invitrogen Korea, Seoul, South Korea) is then added. To observe drug effects, drugs are mixed with medium considering final concentration of each drug. Images were taken using an inverted confocal laser scanning microscope (Nikon Ti-E, Tokyo, Japan) to observe dynamic morphology of G-GSC11 TSs. To quantitate the invasion assay, the maximal area covered by migrating edges of cells were used as a parameter for defining invasiveness (invaded area at certain time/TS area at initial time×100). Data are analyzed through image analysis software ImageJ (NIH, Bethesda, Maryland, USA).

7. Uptake of ^{18}F-fluorodeoxyglucose (FDG)

GSC11 cells were plated on 12-well plates with $3x10^5$ cells per well for 24 hrs. The medium was changed to a glucose-free DMEM medium (Gibco, Invitrogen Korea, Seoul, South Korea) containing approximately 0.5 uCi of ^{18}F-FDG, followed by incubation for 15min. The cells were washed with phosphate-buffered saline 3 times and 0.1 mL of lysis buffer was added to each

well. The lysed cells were then harvested to measure the amount of radioactivity by Wallac 148 Wizard 3 gamma-counter (PerkinElmer Life and. Analytical Science, Shelton, CT, USA). The radioactivity measured was normalized to protein content.

8. Gene expression microarray and gene ontology analysis

Total RNA was extracted from 100mg of tissue using a Qiagen miRNA kit according to the manufacturer's protocol. Expression profiles of drug treated groups and control were obtained using Illumina HumanHT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA,USA) Data were log2 transformed and normalized with quantile normalization method using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team. Genes showing minimal variation across the set of arrays were excluded from the analysis. Genes whose expression differed by at least 1.5 fold from the median in at least 20% of the arrays were retained. Gene set comparison tools provided by BRB-ArrayTools was used for Gene ontology analysis.

9. Orthotopic xenograft animal model

Four-to 8-week-old male athymic nude mice (Central Lab. Animal Inc., Seoul, South Korea) were used for experiments. Mice were housed in micro-isolator cages under sterile conditions and observed for at least 1 week before study initiation to ensure proper health. Lighting, temperature, and humidity were controlled centrally. All experimental procedures were approved by Yonsei University College of Medicine Institutional Animal Care and Use Committee. Mice were anesthetized with a solution of Zoletil® (30 mg/kg; Virbac Korea, Seoul, South Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, South Korea) delivered intraperitoneally. GSC11 were implanted into the right frontal lobe of nude mice using a guide-screw system within the skull. Mice received 5×10^5 cells via a Hamilton syringe Dongwoo Science Co., Seoul, South Korea) inserted to a depth of 4.5 mm. Then, 15mg/kg of HL156A 30mg/kg of TMZ and their combination were administrated to mice. HL156A was given to mice by oral administration every other day until the duration of the experiment and TMZ was administrated intraperitoneally, for 5 days from the day of tumor sphere injection. Five animals per group were injected. The body weight of mice was checked every other day.

If the weight decreased more than 15 % compared to the original body weight, mice were euthanized according to protocol. When mice died, we carefully removed mouse brains and observed gliomagenesis after generating Hematoxylin-Eosin stained slides.

10. Statistical analysis

Data were analyzed using SPSS for Windows, Version 12.0 (SPSS Inc., Chicago, IL, USA) and STATA Statistical Software Release 13(StataCorp LP., College Station, TX, USA). Student T-test was used for comparison of mean of viability and FDG uptake of agent treated cells. Kaplan-Meier survival curves and log-rank statistics were employed for survival analysis. Results were considered statistically significant when $P<0.05$.

III. RESULTS

1. The chemical structure of HL156A

The chemical structure of HL156A (N-(N-(4-(trifluoromethoxy) phenyl) carbamimidoyl) pyrrolidine-1-carboximidamide acetate) is depicted in Figure 1. It is a derivative of biguanide with high bioavailability. In contrast to metformin having limited brain-blood barrier permeability and low bioavailability, it enters the cell independent of OCT1 transporter and highly penetrates to brain.

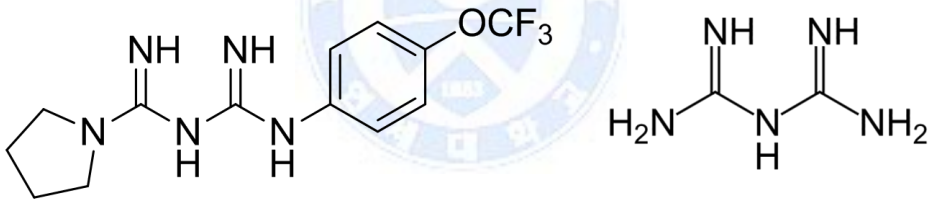


Figure 1. The structure of HL156A (N-(N-(4-(trifluoromethoxy) phenyl) carbamimidoyl) pyrrolidine-1-carboximidamide acetate, left) and metformin (right). The compound contains a central part of metoformin, the biguanide, in its composition.

2. The effect of HL156A, TMZ and combination treatment on cell viability

Using GBM cancer stem cell line X01 and GSC11, I examined cell viability on the treatment of the HL156A, TMZ and combined HL156A and TMZ by MTT assay. The sublethal dose treatment of the agents showed minimal effect on the cells. (Figure 2A). I used sublethal dose of each drug as we pursued specific cellular phenomenon without killing cells. I adopted 15μM of HL156A and 500μM of TMZ for further experiments (Figure 2B).

3. Effect of HL156A, TMZ and combination treatment on AMPK and mTOR pathway of GSC11 and X01 TSs

Generally, it is known that biguanide works as AMPK agonist and consequently, mTOR inhibition is followed. However, this was not the case in GBM-TS. mTOR inhibition by AMPK activation was not observed in GSC11 and X01 cells.

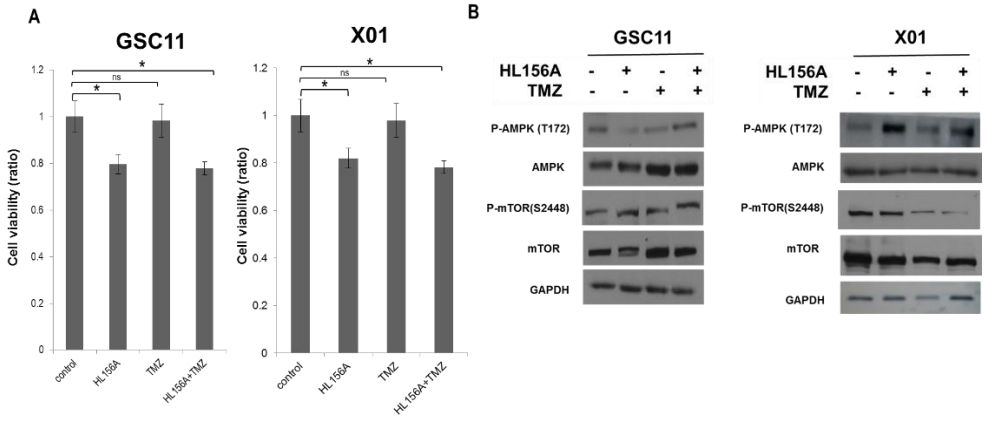


Figure 2. The effect of HL156A, TMZ and combination treatment on stem cell viability. (A) The treatment showed minimal effect on the cell viability. (B) The effect of drugs on AMPK and mTOR pathway. Even though it is known that biguanide works as AMPK agonist and consequent mTOR inhibition is followed, this was not observed in GSC11 and X01 TSs (*: statistically significant, ns : statistically not significant, TMZ: temozolomide, TS: tumorsphere).

4. The effect of HL156A, TMZ and combination treatment on the stemness of TSs

HL156A seems to decrease stemness of GSC11 and X01, proven by neurosphere formation assay (Figure 3A and 3B) and western blotting of

stemness markers such as nestin, CD133, Sox-2, Notch 1, Notch 2 and Oct3/4 (Figure 3C). The number of neurosphere was markedly decreased on the treatment of HL156A and this inhibitory effect was most prominent in the combination treatment. Decreased expression of CD133, OCT 3/ 4, Sox-2, Notch 1 and Notch2 was observed in either GSC11 or X01 (Figure 3C).

5. The effect of HL156A, TMZ and combination treatment on neuro-glial differentiation of GSC11 and X01 TSs

Either HL156A or TMZ did not promote neuro-glial differentiation as the expression of neuronal marker such as olig2, Tuj1, and GFAP were not changed (Figure 4). Thus, it is unlikely that the therapeutic effect of HL156A is through differentiating tumor cells, which is one of proposed strategies for targeting stem cells.

6. The effect of HL156A, TMZ and combination treatment on the invasive property of GSC11 TSs

In 3D invasion assay with collagen I matrix, TS treated with HL156A, TMZ and combination exhibited less degree of invasiveness than control as depicted (Figure 5A, cells with drug treatment at the time of implantation). The

effect was most obvious when the combination treatment was given. It was not because of drug effects on the cell viability as I also assessed the cell viability during the experiment, with no significant cell death. To recapitulate real clinical situation where a drug is administered to the patient after the tumor formation, I performed the same assay but the treatment of drug was given 12 hrs after implantation (Figure 5B). Even in this setting, the inhibitory effect of invasion was most prominent when the combination treatment was given. As several groups reported that EMT related markers were upregulated in GBM and the acquisition of mesenchymal traits by cancer cells undergoing EMT has been reported to be related with the acquisition of a stem cell program ,[19,25] the expression of EMT related markers such as β-catenin, zeb1, N-cadherin and snail was assessed, revealing the expression of β-catenin, zeb1, and N-cadherin was diminished on the treatment of HL156A and combination treatment, suggesting that the agent may perturb the EMT related pathway of the GBM-TS (Figure 5C).

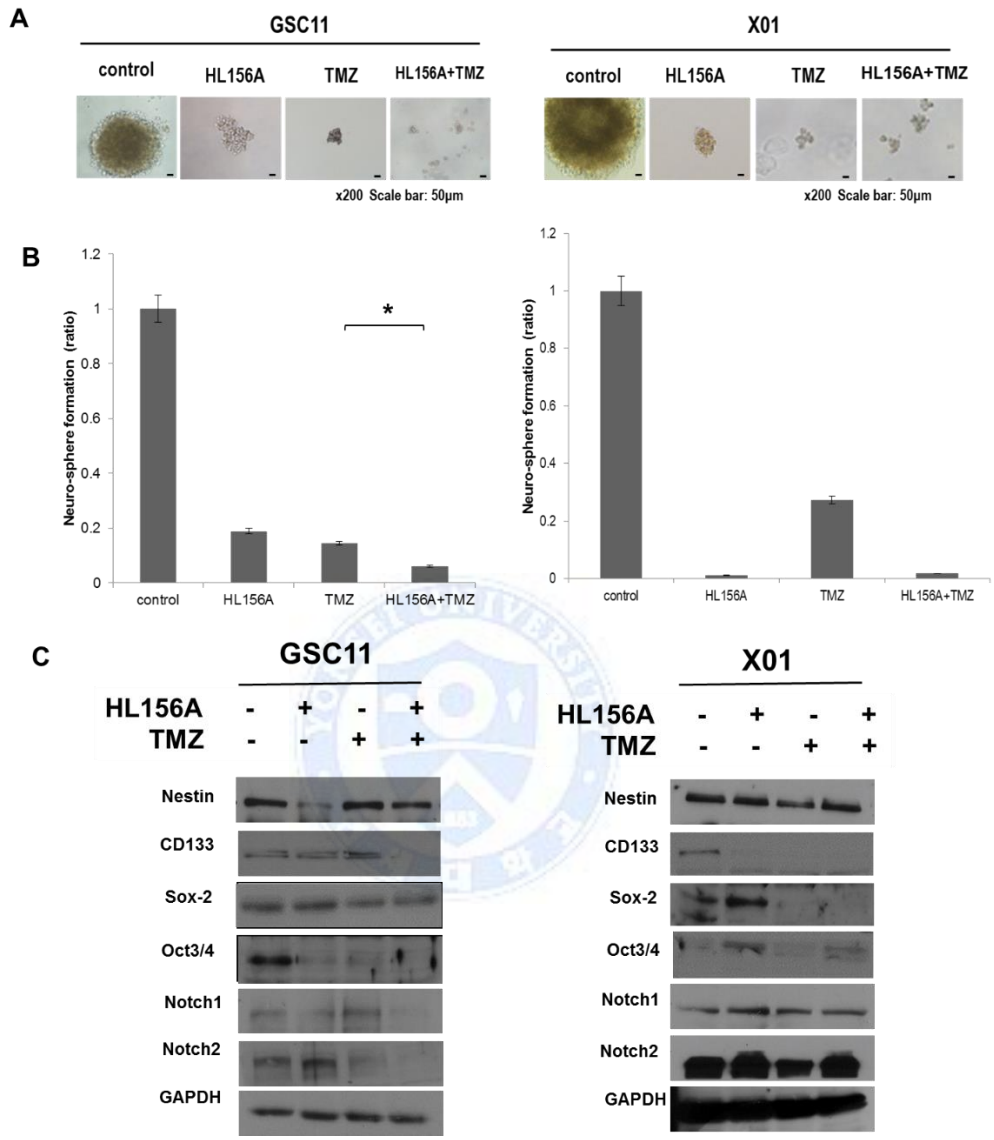


Figure 3. The effect of HL156A, TMZ and combination treatment on stemness of GBM-TSs. (A, B) Drug effect on the stemness of GSC11 and X01 TSs was assessed by the neurosphere formation assay. The number of

neurosphere was decreased on the treatment of HL156A and combined HL156A and TMZ. (C) The expression of stemness markers, such as CD133 and Sox, were decreased in the combination treatment group (*: Statistically significant).

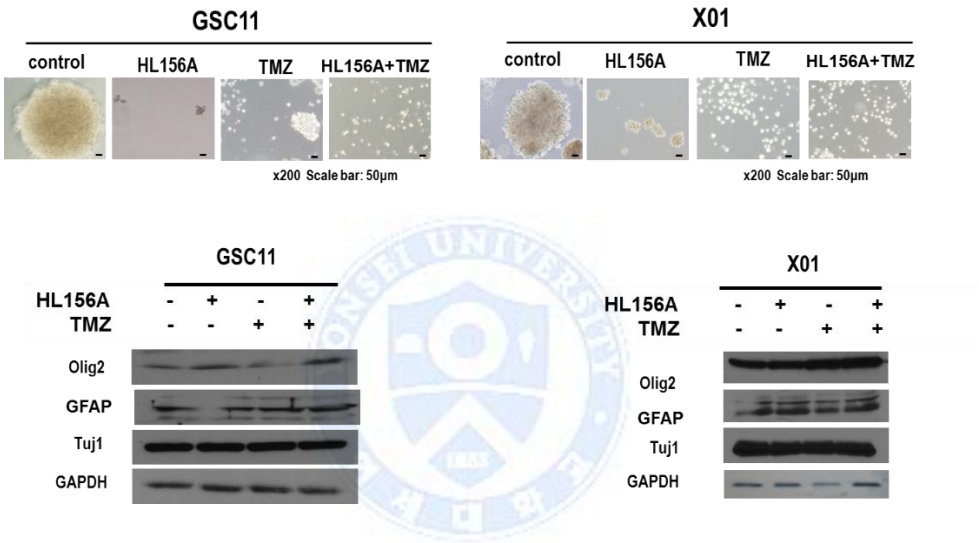


Figure 4. The effect of HL156A, TMZ and combination treatment on neuroglial differentiation of GBM-TSs. The treatment of HL156, TMZ and combination of them did not affect neuro-glial differentiation of GSC11 TSs.

7. Assessment of cellular metabolism by analyzing ^{18}F-FDG uptake

In order to assess the effect of drugs on the cellular metabolism, ^{18}F-FDG uptake was examined. The uptake of ^{18}F-FDG was decreased as cells were

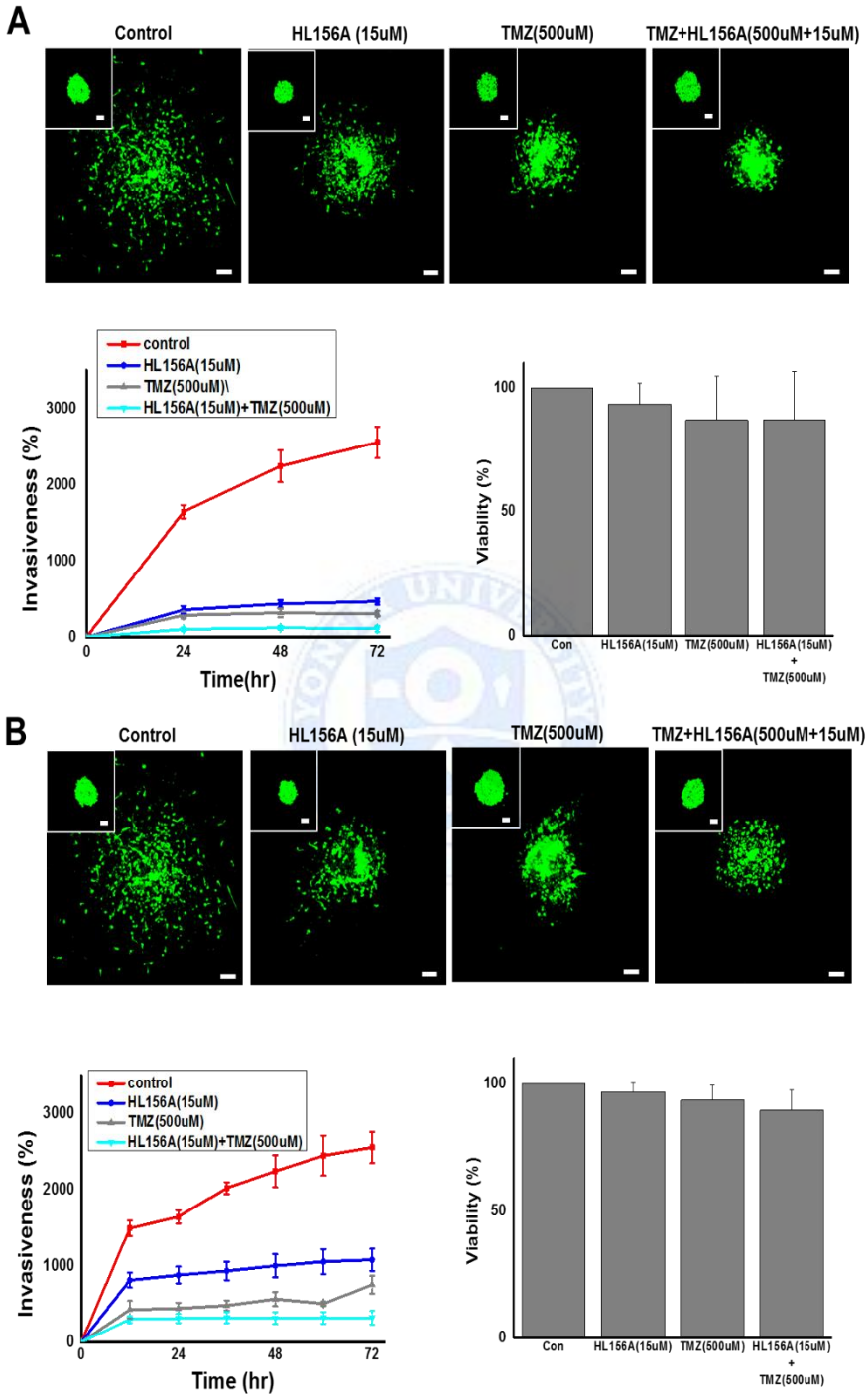
treated with drugs and the decrease was most prominent when the combination treatment was given. Thus, HL156A seems to decrease cellular metabolism and the degree of inhibition is most obvious in HL156A and TMZ combination treatment (Figure 6).

8. Gene ontology analysis of microarray data

Transcriptome analysis by Illumina HumanHT-12 v4 Expression BeadChip was acquired and heatmap with hierarchical clustering was generated (Figure 7). There are genes differentially expressed when the combination treatment was given compared to control group, among which several down-regulated genes such as FBLN7, an adhesion molecule that interacts with extracellular matrix, Lyn, a molecule known to regulate cell migration, and LAMA4, a laminin were included. I also analyzes Gene Ontology gene sets for differential expression among TMZ and combination treatment using BRB-Array tool. When compared with TMZ single treatment, the combination treatment showed differentially expressed gene sets that include those related with cell adhesion, cell migration, cell motion and regulation of cell adhesion (Table 1).

9. The effect of HL156A on the xenografted tumor growth

Finally, the effect of drugs on the tumors of orthotopic xenograft mice was analyzed. After sacrificing animals, brains of animals were taken out and stained. Although combination treatment did not prevent the formation of the mass, the size and extent of mass was limited in the combination-treatment group mice (Figure 8A). The survival of the orthotopic xenographfted mice (n=5 per group) were analyzed, revealing that the treatment of combination of HL156A and TMZ had a benefit in overall survival of animals (p=0.000, log rank test, Figure 8B).


A
Control
HL156A (15uM)
TMZ(500uM)
TMZ+HL156A(500uM+15uM)
control
HL156A(15uM)
TMZ(500uM)\
HL156A(15uM)+TMZ(500uM)
Invasiveness (%)
3000
2000
1000
0
0
24
48
72
Time(hr)
Viability (%)
100
50
0
Con
HL156A(15uM)
TMZ(500uM)
HL156A(15uM) + TMZ(500uM)
B
Control
HL156A (15uM)
TMZ(500uM)
TMZ+HL156A(500uM+15uM)
control
HL156A(15uM)
TMZ(500uM)
HL156A(15uM)+TMZ(500uM)
Invasiveness (%)
3000
2000
1000
0
0
24
48
72
Time (hr)
Viability (%)
100
50
0
Con
HL156A(15uM)
TMZ(500uM)
HL156A(15uM) + TMZ(500uM)

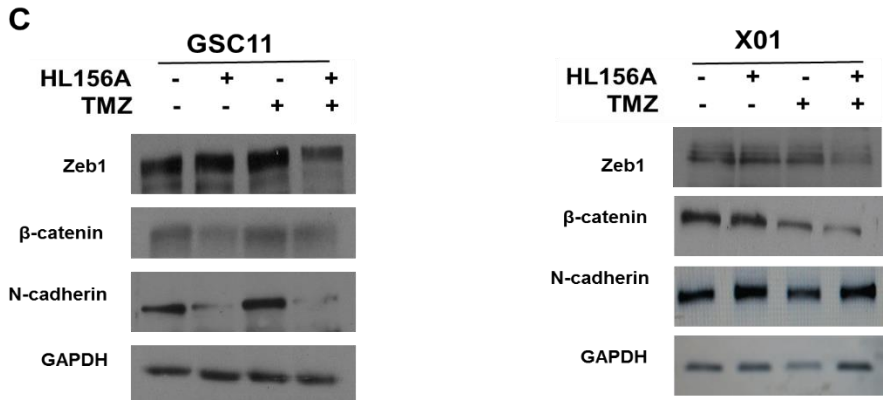


Figure 5. The effect of HL156A, TMZ and combination treatment on the invasive property of GBM-TSs. (A, B) The treatment of HL156A and combination of HL156A and TMZ showed decreased invasiveness on 3D collagen matrix invasion assay (A: drug treatment at the time of cell implantation, B: drug treatment after 12 hrs of cell implantation). (C) Markers related with EMT pathway were altered on the treatment of HL156A. Expression of β-catenin, zeb1 and N-cadherin was decreased on the treatment of HL156A and combination of HL156A and TMZ.

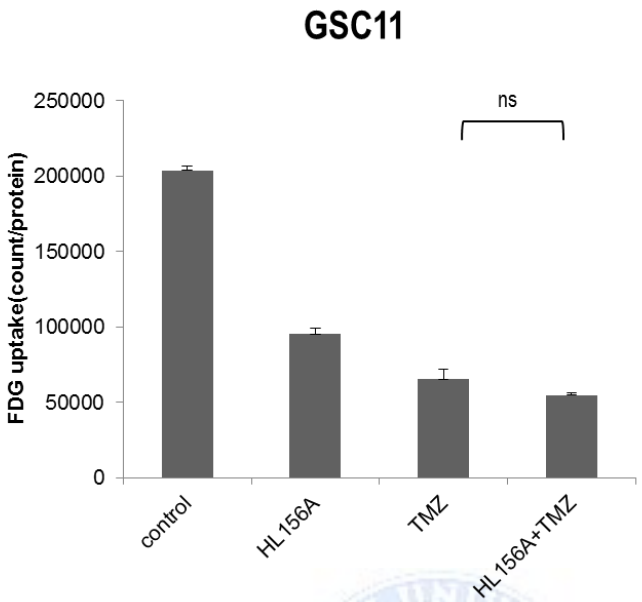


Figure 6. F^{18}-FDG uptake was markedly decreased in HL156A, TMZ and combination treatment. The decrease in FDG uptake was most prominent in combination treatment group, suggestive of low metabolic status (ns : statistically not significant).

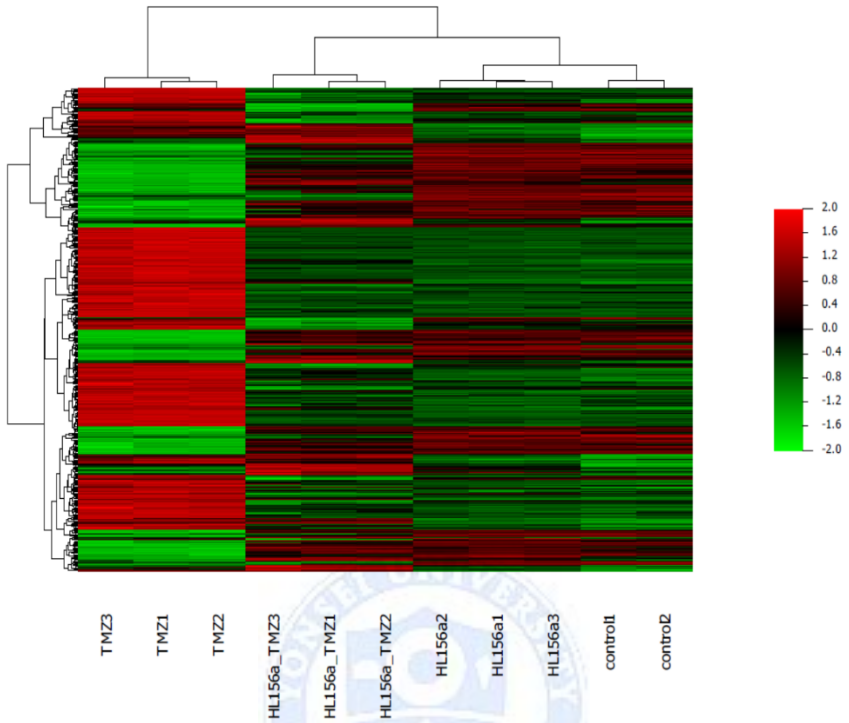


Figure 7. High throughput gene expression microarray. Sets of genes were differentially expressed in the combination treatment compared with the control and single treatment of either HL156A or temozolmide.

Table 1.Results of gene ontology analysis

	GO category	GO ontology	GO term	Number of genes	LS* permutation p-value	Efron-Tibshirani's GSA test p-value
1	GO:0007155	BP	**cell adhesion**	88	0.00003	0.04 (+)
2	GO:0022610	BP	**biological adhesion**	88	0.00003	0.04 (+)
3	GO:0048699	BP	generation of neurons	96	0.00008	< 0.005 (-)
4	GO:0050839	MF	**cell adhesion molecule binding**	22	0.00023	0.11 (+)
5	GO:0001558	BP	regulation of cell growth	29	0.00023	< 0.005 (+)
6	GO:0045595	BP	regulation of cell differentiation	92	0.00029	0.095 (+)
7	GO:0048870	BP	**cell motility**	93	0.00035	< 0.005 (+)
8	GO:0051674	BP	localization of cell	93	0.00035	< 0.005 (+)
9	GO:0016477	BP	**cell migration**	92	0.00041	< 0.005 (+)
10	GO:0034330	BP	cell junction organization	12	0.00044	< 0.005 (-)
11	GO:0030182	BP	neuron differentiation	86	0.00046	< 0.005 (-)
12	GO:0000902	BP	cell morphogenesis	82	0.00047	0.205 (+)
13	GO:0016337	BP	**single organismal cell-cell adhesion**	28	0.0005	< 0.005 (+)
14	GO:0009611	BP	response to wounding	68	0.0005	0.07 (+)
15	GO:0016192	BP	vesicle-mediated transport	73	0.00061	0.335 (+)
16	GO:0048666	BP	neuron development	67	0.00069	< 0.005 (-)
17	GO:1903035	BP	negative regulation of response to wounding	9	0.00072	< 0.005 (+)
18	GO:0005178	MF	**integrin binding**	13	0.00072	< 0.005 (+)
19	GO:0006897	BP	endocytosis	34	0.00074	0.435 (+)
20	GO:0030155	BP	**regulation of cell adhesion**	34	0.00077	< 0.005 (+)
21	GO:0045216	BP	cell-cell junction organization	11	0.00077	< 0.005 (-)
22	GO:0034097	BP	response to cytokine	55	0.00078	0.145 (+)
23	GO:0007160	BP	cell-matrix adhesion	12	0.0008	< 0.005 (-)
24	GO:0031348	BP	negative regulation of defense response	10	0.00082	< 0.005 (+)
25	GO:0098602	BP	**single organism cell adhesion**	32	0.00096	< 0.005 (+)
26	GO:0016049	BP	cell growth	38	0.00099	< 0.005 (+)

27	GO:0030030	BP	cell projection organization	74	0.001	0.14 (-)
28	GO:0032102	BP	negative regulation of response to external stimulus	14	0.00101	0.12 (+)
29	GO:0032989	BP	cellular component morphogenesis	86	0.00115	0.245 (+)
30	GO:0034329	BP	cell junction assembly	10	0.00117	< 0.005 (-)
31	GO:0050767	BP	regulation of neurogenesis	41	0.00133	0.21 (+)
32	GO:0051093	BP	negative regulation of developmental process	51	0.00159	< 0.005 (+)
33	GO:0023056	BP	positive regulation of signaling	76	0.00169	< 0.005 (+)
34	GO:0010647	BP	positive regulation of cell communication	77	0.00189	< 0.005 (+)
35	GO:0031175	BP	neuron projection development	62	0.00192	0.08 (+)
36	GO:0001952	BP	**regulation of cell-matrix adhesion**	5	0.00197	< 0.005 (-)
37	GO:0031589	BP	**cell-substrate adhesion**	19	0.00206	0.03 (-)
38	GO:0043405	BP	regulation of MAP kinase activity	18	0.00226	< 0.005 (+)
39	GO:0050728	BP	negative regulation of inflammatory response	7	0.00245	< 0.005 (+)
40	GO:2001237	BP	negative regulation of extrinsic apoptotic signaling pathway	9	0.00251	< 0.005 (+)
41	GO:0000904	BP	cell morphogenesis involved in differentiation	64	0.00253	0.08 (+)
42	GO:0043588	BP	skin development	24	0.0026	0.25 (+)
43	GO:0007264	BP	small GTPase mediated signal transduction	23	0.00263	0.21 (-)
44	GO:0009897	CC	external side of plasma membrane	17	0.00271	0.055 (+)
45	GO:0051216	BP	cartilage development	13	0.00324	< 0.005 (+)
46	GO:0045664	BP	regulation of neuron differentiation	33	0.00344	0.21 (+)
47	GO:0007266	BP	Rho protein signal transduction	7	0.00345	< 0.005 (-)
48	GO:0043406	BP	positive regulation of MAP kinase activity	11	0.00348	< 0.005 (-)
49	GO:0051960	BP	regulation of nervous system development	44	0.00351	< 0.005 (+)

50	GO:0061448	BP	connective tissue development	17	0.00353	< 0.005 (+)
51	GO:0033559	BP	unsaturated fatty acid metabolic process	6	0.00368	< 0.005 (+)
52	GO:1903034	BP	regulation of response to wounding	31	0.00371	0.145 (+)
53	GO:0006909	BP	phagocytosis	11	0.00395	0.34 (+)
54	GO:0048864	BP	stem cell development	16	0.00416	< 0.005 (+)
55	GO:0061024	BP	membrane organization	44	0.00425	0.035 (+)
56	GO:0006954	BP	inflammatory response	34	0.00427	0.145 (+)
57	GO:0030054	CC	cell junction	80	0.00438	0.175 (-)
58	GO:0030098	BP	lymphocyte differentiation	13	0.00448	0.4 (+)
59	GO:0048771	BP	tissue remodeling	18	0.00453	0.15 (-)
60	GO:0022407	BP	**regulation of cell-cell adhesion**	13	0.00458	< 0.005 (+)
61	GO:0032990	BP	cell part morphogenesis	59	0.00479	0.14 (-)

Differentially expressed gene sets between TMZ and combination treatment. Expression of several gene sets related cellular adhesion and migration (bold highlight) was significantly different between two groups (* LS : least square).

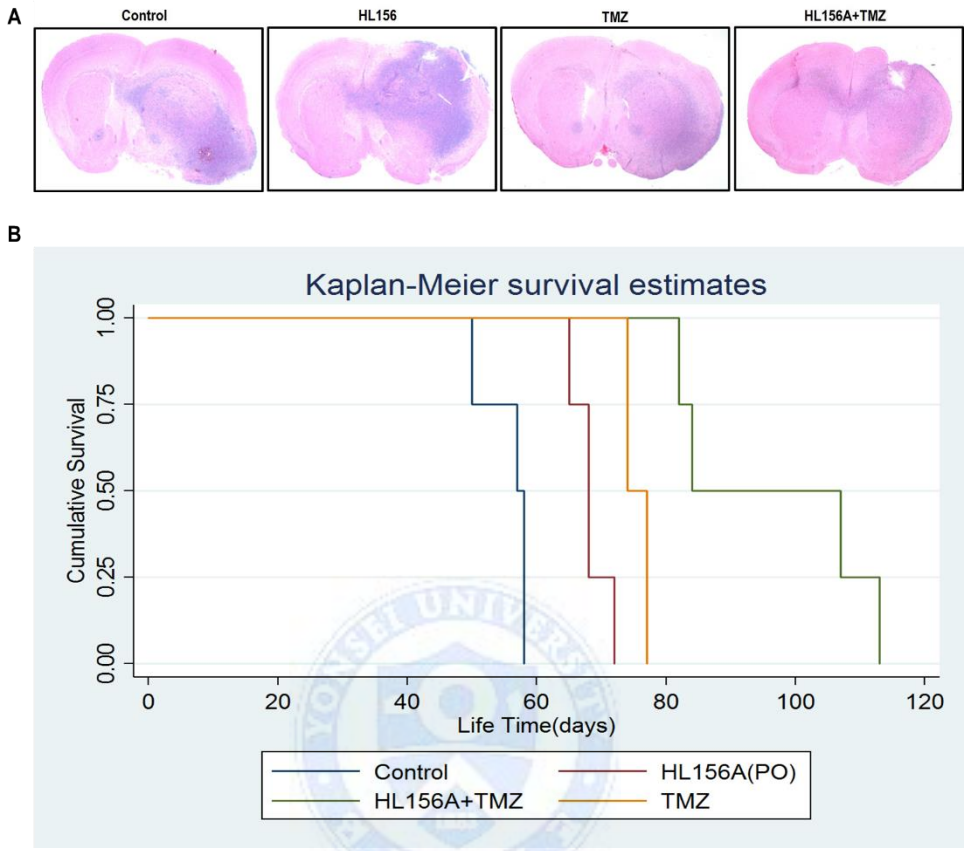


Figure 8. The effect of HL156A and combined HL156A and TMZ on the xenografted tumor growth and survival of animal. (A) Tumors from HL156A, TMZ, and combined HL156A and TMZ treated animals were stained and examined. HL156A and combination treated animal showed less irregular margin than that of control. Especially, combination treatment group showed tumors with decreased volume. (B) Survival of the combination treatment group was significantly increased (p=0.0000).

IV. DISCUSSION

In spite of recent progress in the understanding of GBM, survival of patients is still very limited. Even after a complete resection was achieved at the time of operation, it tends to recur and takes patient's life. A new strategy against the disease is required, accordingly. Targeting GBM-TS and identification of key molecules related with the unique properties may be a reasonable approach as many reports showed chemo- and radio-resistance of GBM-TS that potentially cause recur of the disease.

In the present study, I examined the effect of HL156A, TMZ and combination treatment on the stemness and invasive property of GBM-TS. The combination treatment of new agent HL156A and conventional TMZ, inhibited stemness and invasive properties of GSC11, and this effect seemed to be related with the alteration of EMT related markers. Similar additive effect of metformin with conventional therapy was found on the breast cancer stem cells model.[34] This group showed an additive effect of metformin in the cytotoxic effect of hyperthermia and this was via the activation of AMPK and inactivation of mTOR.[34] Furthermore, it was shown that metform killed and radiosensitized

cancer cells and preferentially killed cancer stem cells of breast.[35] This kind of effect was also known in GBM. One group reported metformin plus TMZ-base chemotherapy as an adjuvant treatment for WHO grade III and IV malignant gliomas.[36] And another group specifically target cancer stem cells of GBM by metformin plus sorafenib.[37] The possible explanation of the superior result of survival in the combination treatment group in this study may be contributed to 1) the tumor cytotoxic effect of TMZ in addition to the deteriorated cellular energy metabolism environment induced by HL156A 2) the additive inhibitory effect of TMZ and HL156A on the invasive property of TSs, which results in the less aggressive tumor phenotype. Given the heterogeneity of cancer cells, targeting GBM-TSs by general metabolic stress seems to be a reasonable approach as it may provide less favorable environment to the metabolically active tumor cells generally. Although a well-known biguanide metformin may have similar effect as HL156A, the delivery of metformin to the brain is limited. The new compound overcomes this shortcoming and generally shows high bioavailability. Further study on this combination treatment strategy with this new compound in addition to the conventional therapy in the treatment of malignant tumor need to be performed.

The cellular mechanism underlying the effect of HL156A seems distinctive in that it did not show expected AMPK activation and consequent mTOR inhibition pathway in the setting of GBM, as in this study. Recently, Liu et al. reported that AMPK is activated in the glioblastoma and the anti-proliferative effect of metformin is AMPK independent. Degrading cdc25 through proteasome and/or increasing PRAS40-RAPTOR interaction were suggested as inhibitory effectors for mTOR, independent of AMPK in this study.[38] The present study also confirmed increased basal AMPK expression in GSC11 and X01 TSs and similarly, AMPK activation and consequent mTOR inhibition was not obvious in the inhibitory effect of HL156A and combination treatment on the GBM-TSs. It was not possible to verify these phenomena in the gene expression microarray data as most of genes were filtered out during the quality-controlprocess. Interestingly, expression of DNA-Damage-Inducible Transcript 4 (DDIT4), having inhibitory effect on mTOR1, was significantly increased in combination treatment group. Elucidation of this increase with regards to TS- inhibitory effect of combination treatment need to be sought, as growth inhibitory effect of HL156A was not obvious in the tested concentration. Although it was not obvious in the concentration up to 200μM,

it is still worth assessing effect of drugs on GBM-TS in higher dose range with regards to the activation of AMPK and subsequent mTOR inhibition. In addition, the direct experimental assessment of OXPHOS pathway in GBM-TS in the setting of inhibitory effect of drugs need to be performed for the further study.

Another unique aspect of the result of this work is that the inhibitory effect on the invasive property of GBM-TSs may be related with the alteration of EMT related markers. The evidence of EMT pathway in the pathogenesis of GBM expansion and invasion is not robust at this point. Even though there exists a similarity in the process of GBM invasion and immature neuron migration during embryonic development, little is known in the process and direct evidence of relation of invasive property and EMT is still lacking. Nevertheless, several evidence suggested similar molecular alteration happen to be exist in the GBM pathophysiology and outcome after inhibiting this pathway need to be studied to identify the role of this pathway in the invasive property of this fatal disease. Similar experiment with targeting EMT pathway by siRNA or shRNA may help.

Even after identifying TMZ responsive group, the patients succumb to death by relapse of the disease. There were several strategies proposed for overcoming therapeutic limitation of GBMs, most of them still not successful. Due to the intrinsic infiltrative nature of the tumor into the normal brain tissue, the complete resection during the operation seems impossible. Thus, proper adjuvant therapy for the potential remaining cancer cells including cancer stem cells seems crucial to overcome the detrimental fate of the disease. Targeting of GBM-TSs as a new strategy need to be extensively evaluated in this context. Caveats of the theory is limited information regarding the identification of GBM specific stem cells, which makes sorting out the cells for the experiment limited. For example, there still are controversies on the surface markers that defined cancer stem cells. Furthermore, some properties of GBM is able to be explained by the clonal evolution model of the disease, another axis of tumor heterogeneity. Heterogeneity defined by spatial structure of tumor can be explained better by the clonal evolution model. As combined model also was suggested, more evidence need to be accumulated to understand this specific nature of the disease. General intracellular energy metabolism alteration seems to be a reasonable therapeutic approach as it is able to target neoplastic cells by

this strategy in both models. Accordingly, usage of this newly developed agent HL156A combined with conventional TMZ for the targeting GBM-TSs, need to be further evaluated as a new strategy against the disease.

V. CONCLUSION

I assessed an effect of a newly developed biguanide, HL156A on the properties of GBM-TSs and survival of orthotopic xenograft animals, to assess the feasibility of this newly developed agent for the treatment of GBM, alone or combined with conventional therapeutic agent TMZ. HL156A, especially combined with TMZ exhibited inhibitory effect on the stemness of GBM-TSs, proven by sphere formation assay and the assessment of marker expression, without affecting viability of cells. Secondly, HL156A did not promote neuro-glial differentiation. Thirdly, the invasive properties of GBM-TSs were inhibited by the combination treatment, compared with the control and TMZ alone treated groups in 3-dimentional collagen matrix invasion assay. Fourthly, treatment of combined HL156A and TMA seems to repress epithelial-mesenchymal transition (EMT) related gene expression. Fifthly, gene ontology class comparison of TMZ and combination treatment group reveals altered expression of gene sets involving cellular adhesion and migration. Finally, the combined treatment of HL156A and TMZ showed survival benefits in the orthotopic xenograft mouse model. In conclusion, targeting of GBM-TSs by the combination of HL156A and TMZ, through the inhibition of stemness and

invasion properties of GBM- TSs, can be a novel candidate for the treatment of GBM.

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ABSTRACT (IN KOREAN)

새롭게 개발된 바이구아나이드 화합물에 의한 교종 종양구 억제 효과

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최 준 정

교모세포종(glioblastoma)은 치명적인 중추신경계의 병으로, 최근 이 분야의 연구의 발전에도 불구하고 제한적인 생존율을 보이고 있으며, 따라서 새로운 치료전략의 수립이 필요한 병이다.최근 악성종양의 치료제로 바이구아나이드(biguanide)의 역할이 대두되어 연구되어 있으며, 일부에서 바이구아나이드의 종양구에 대한 효과가 보고되고 있다. 본 연구에서 새롭게 개발된 바이구아나이드 화합물 HL156 의 교모세포종의 치료제로서의 적합성을 판정하기 위하여, HL156A 단독, 그리고 기존치료제 테모졸로마이드와의 병합 시

교모세포종 종양구의 줄기세포성과 침윤성에 대한 효과와 동소이종이식 마우스에서의 생존율을 평가하였다. HL156A 와 테모졸로마이드와의 병합투여의 교모세포종 종양구의 줄기세포능 저하영향을 종양구 형성실험과 마커 발현을 통해 확인하였으며, 삼차원 콜라겐 기질 침윤분석을 통하여 병합투여가 교모세포종 종양구의 침윤성을 억제하는 것을 확인하였다. 또한, HL156A 의 단독, 혹은 병합투여는 상피-간질이행 관련 유전자 발현을 억제하는 효과를 나타내었다. 유전자 온톨로지 분석을 통하여, 병합투여시 테모졸로마이드 단독투여 때보다 세포 접착 및 세포 이동과 관련한 유전자세트의 변화가 있음을 확인하였다. 마지막으로, HL156A 의 테모졸로마이드와의 병합투여는 동소 이종 이식마우스 모델에서 생존의 유의한 생존 증가를 나타내었다. 결론적으로, 개발된 바이구아나이드 화합물은 교모세포종 종양구의 줄기세포능과 침윤성을 억제하여 교모세포종의 새로운 치료제로 사용될 수 있으며 이에 대한 평가가 추후로 이루어져야 한다.

핵심되는 말: 바이구아나이드, 교모세포종, HL156A, 침윤성, 줄기세포능, 종양구