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A Dissertation for the Degree of Doctor of Philosophy

**Anti-Cancer Mechanisms of MICA
Nanoparticle or PDE4D Inhibitor
in Human Cancer Cells**

암세포에서 MICA 나노입자와 PDE4D 억제제의
종양 억제 기전 연구

By

Tae-Wook Kang

August 2016

Program in Zoonotic Animal Disease,
College of Veterinary Medicine,
Graduate School of Seoul National University

ABSTRACT

Anti-Cancer Mechanisms of MICA Nanoparticle or PDE4D Inhibitor in Human Cancer Cells

Tae-Wook Kang

Program in Zoonotic Animal Disease,
College of Veterinary Medicine,
Graduate School of Seoul National University

Supervisor: Kyung-Sun Kang, D.V.M., Ph.D.

Glioblastoma multiforme (GBM) and breast cancer are the most prevalent malignant tumors in adults, and both exhibit high fatality rates due to their tumorigenic potentials and limitations of cancer therapy. In this study, I investigated on tumor suppressive and immunostimulatory effects using novel small molecule, CG500354, and natural compound, STB-HO.

In the first part of this study, I performed forced differentiation of cancer cells, which is a recently developed approach in developing a cancer therapy. I demonstrated that the novel phosphodiesterase-4 subtype D (PDE4D) inhibitor, CG500354, induces growth arrest and neural differentiation in GBM-derived cells by triggering the activation of cAMP/PKA signaling pathway. Treatment of CG500354 regulated cAMP/PKA signaling pathway by interrupting PDE4 interference and up-regulating phosphorylated protein kinase A (p-PKA) and phosphorylated CREB (p-CREB). Furthermore, PDE4D inhibitor suppressed the expression level of cyclin B1, while up-regulating p21 and p27, which are known to be associated with growth arrest. I next investigated whether PDE4D inhibitor affect neural differentiation of GBM. GFAP, an astrocyte marker, and Tuj-1, a neuronal marker, were significantly increased with p53 expression level in GBM while the expression of nestin, a neural progenitor marker was decreased. These results suggest that CG500354 may play crucial roles in neural differentiation and growth arrest through regulation of cell-cycle-related and neural differentiation markers.

In the second part of this study, I investigated on the regulation of interactions between tumor cells and anti-tumor immune cells by treating with mica nanoparticle, STB-HO. Its efficacy and mechanisms in treating various types of tumor are less verified and the mechanistic link between anti-tumor and immunostimulatory effects has not been elucidated. STB-HO was orally administered into MCF-7 xenograft model. The growth of MCF-7 cell in xenograft model was significantly suppressed, whereas STB-HO did not directly affect the proliferation and apoptosis *in vitro*. Thus, I observed the interactions of MCF-7 and macrophage, dendritic cells (DCs) and natural killer (NK) cells after STB-HO

treatment to investigate the discrepancy between *in vivo* and *in vitro*. The MHC class I, which is known as an inhibitory factor for NK cell-mediated anti-tumor effect, was down-regulated approximately by 10% when treated with STB-HO. Thus, I induced macrophage-like cells from THP-1 and dendritic cells from CD14⁺ monocytes to analyze the functional alteration of STB-HO. I observed that IL-12, known for its contribution in NK cell cytotoxicity against tumor cell, was consistently increased by STB-HO treatment. I next isolated NK cells from human blood and performed cytotoxicity test by co-culturing NK cells with fluorescence-labeled MCF-7. The dead cell population of MCF-7 rose up to 2% to 7% and additional STB-HO treatment on co-culture condition rose dead cell proportion up to 16-17% along with the elevation of significant IFN- γ expression. I observed that STB-HO not only increased susceptibility of MCF-7 cells to immune cells, but also stimulated immunocytes in tumor microenvironment to eliminate cancer cells.

Taken together, these findings provide insights that CG500354 activated cAMP/PKA signaling pathway by blocking PDE4D, which triggered neural differentiation and growth arrest of glioblastoma-derived cancer cells. STB-HO regulated the interaction of tumor with its immune microenvironment by enhancing the functions of NK cell, macrophage and DCs to attack tumor cells by secreting IFN- γ and IL-12.

Keywords : Glioblastoma, PDE4D inhibitor, cAMP, Breast Cancer, Mica nanoparticle, Tumor-suppressive effect

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LIST OF ABBREVIATIONS

BMP4	Bone morphogenetic protein 4
cAMP	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding
CSCs	Cancer stem cells
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
EP	E-series of prostaglandin
ERα	Estrogen receptor α
FN	Fibronectin
GBM	Glioblastoma multiform
GFAP	Glial fibrillary acidic protein
HIF1α	Hypoxia-inducible factor 1 α
IL-1	Interleukin-1
MACS	Magnetic-activated cell sorting
MCF-7	Michigan Cancer Foundation-7

MHC class I	Major histocompatibility complex class I
MNC	Mononuclear cell
NF-κB	Nuclear factor-κB
NK	Natural killer
PDE4	Phosphodiesterase-4
PDE4D	Phosphodiesterase-4 subtype D
PGE2	Prostaglandin E2
PKA	Protein kinase A
PLO	Poly-ornithine
RT-PCR	Reverse transcription-polymerase chain reaction
STAT3	Signal transducer and activator of transcription 3
TNF	Tumor necrosis factor
Tuj-1	Neuron Class III β-tubulin
UCB	Umbilical cord blood

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LITERATURE REVIEW

Benefit and limitation of cancer therapeutics

Cancer is one of the leading causes of death in human. Unlike normal cells, the complexity and diversity of cancer pose many challenges to cancer research and therapy due to its long-term proliferative and dividing capability and resistances. Furthermore, the traditional therapies were designed to target not only rapidly dividing cells but also certain normal cells. To confront these, cancer treatment has evolved from relatively non-specific cytotoxic drugs to selective mechanism-based therapeutics, and many other engineering tools and techniques have been created and studied in vitro and in vivo for new treatment strategies (Gu and Mooney, 2016; Vanneman and Dranoff, 2012). Both traditional- and newly developed- therapeutics are being used in clinical trials worldwide (Fig.1)

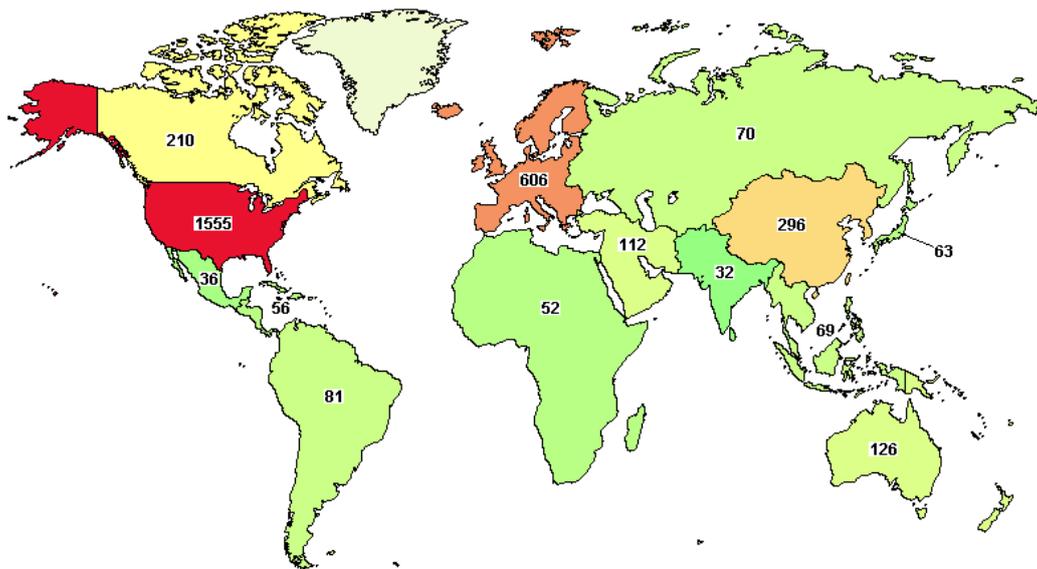
Newly developed anticancer therapeutics such as combination therapy, hormone therapy, immunotherapy and targeted therapy (Buckner et al., 2014; Melero et al., 2015; Sweeney et al., 2014; Vanneman and Dranoff, 2012) not only lead the increase of survival rate in patients, but also improve and redeem the efficacy of traditional cancer treatment such as chemotherapy, radiotherapy and surgical treatment (Nguyen and Tsien, 2013; Veldeman et al., 2008; Weissleder and Pittet, 2008; Zamboni et al., 2012).

Targeted therapies such as antibody drugs and small molecule drugs are similar to other standard chemotherapy drugs which often cannot penetrate nor target the core of tumors, and also cannot give selective action only to the cancer cells. But targeted therapies are different in the way to not only affect the outer cell working, but also regulate the inner cell environment by targeting specific genes, proteins or signal transduction. Recently, these therapies are components of treatment for breast cancer, colorectal cancer and melanoma (Baselga, 2001; Gray-Schopfer et al., 2007; Lee-Hoeflich et al., 2008).

Immunotherapy using monoclonal antibodies, interferons, interleukins and checkpoint inhibitors are designed to enhance or train natural immune system function in the tumor microenvironment against kidney cancer, skin cancer and melanomas. Moreover, immunotherapy may offer the possibility for long-term cancer remission and lower the damage to surrounding normal tissue with less side effects such as alopecia, organ dysfunction and mucositis (Gutterman, 1994; Latchman et al., 2001; Pfeffer et al., 1998; Rosenberg et al., 1988).

The limitations of therapies always exist. To overcome these, novel combination of targeted therapy with immunotherapy is recently being used for several studies (Blank et al., 2011; Hu-Lieskovan et al., 2014). Some targeted therapies manifest immunomodulatory properties and affect diverse aspects of the immune response by improving T cell and NK cell activation. Combining targeted therapies and immunotherapies may offer synergistic responses against cancers and reduce development

of resistances (Bielekova et al., 2006; Korman et al., 2006; Mellman et al., 2011; Vanneman and Dranoff, 2012).



Colors indicate the number of studies with locations in that region



Labels give the exact number of studies

Figure 1. Current ongoing clinical research trials worldwide

(ClinicalTrials.gov, 2016)

PDE4-cAMP/CREB signaling pathway

Cyclic AMP phosphodiesterase-4 (PDE4) has four PDE4 subfamily genes (A–D), from which are comprised of more than 20 different functional isoforms. And it regulates cAMP through their hydrolytic activity (Houslay and Adams, 2003; Li et al., 2011). PDE4 has been suggested as an excellent target because of its cAMP regulating role (Fig.2). PDE4 is selectively eliminated from cancers as a targeted gene in cancer therapy and it indicates that this PDE4 targeted therapy might be a promising approach to tumor therapy (Drees et al., 1993; Merz et al., 1998; Persani et al., 2000; Sengupta et al., 2011; Sette et al., 1994).

The cell growth and proliferative role of cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase (PKA) have been reported (Gottesman and Fleischmann, 1986). It also has been reported that cAMP/CREB signaling pathway related genes act as oncogenes or tumor suppressor (Kirschner et al., 2000; Landis et al., 1989). Moreover, the connection between cAMP level and tumor, and the critical way to regulate cAMP expression towards anti-cancer effect was investigated (Furman and Shulman, 1977; Savai et al., 2010).

The cyclic AMP response element binding (CREB) protein in cAMP pathway and its expression is involved in the regulation of cell replication and differentiation in many tumor types like glioblastoma, breast cancer, melanoma (Chhabra et al., 2007; Dumont et al., 1989; Goldhoff et al., 2008; Melnikova et al., 2010; Zhao et al., 2004). Several papers

investigated a new approach of cancer treatment which suggested that inducing phenotypical alteration of cancers from oncogenic state into a less oncogenic and more differentiated state might be a breakthrough to overcome cancer (Chang and Szabo, 2000; Filleur et al., 2005; Kitamura et al., 1999).

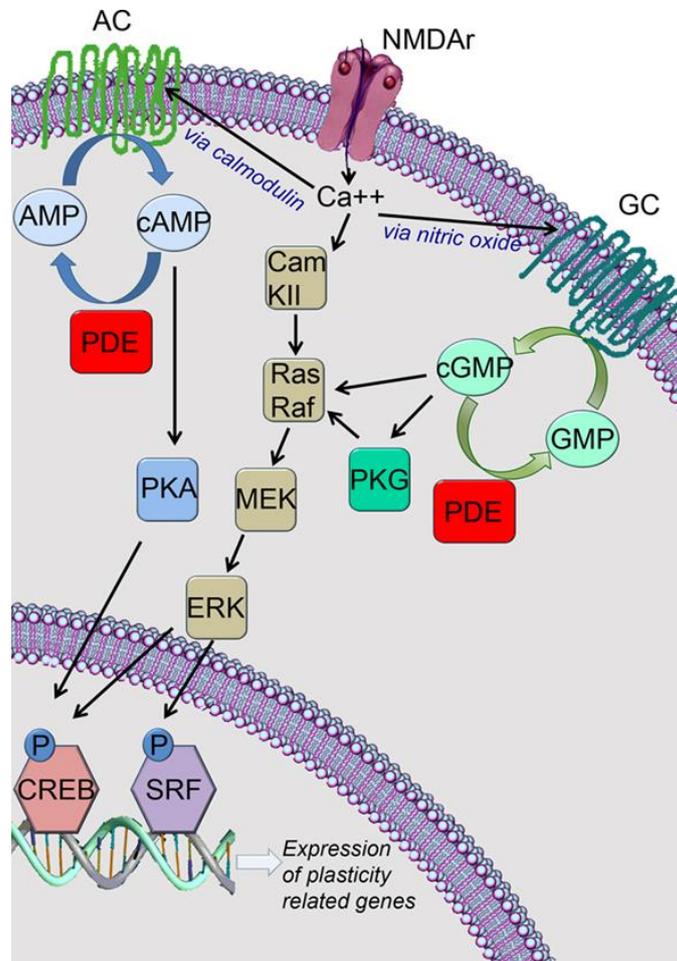


Figure 2. PDE inhibition lead to the increase of cAMP (Medina, 2011)

Immune microenvironment in tumor

The relationship between the tumor and immune system has been a confounding issue. It has been proposed only for a decade that the inflammation plays a critical role in tumorigenesis, and molecular mechanisms based on several evidence have been elucidated (Coussens and Werb, 2002; Grivennikov et al., 2010; Karin, 2006). A role for inflammation in tumorigenesis is partly unraveled, and it has become evident that an inflammatory microenvironment is an essential component of all tumors and could be a key to target cancers, because of its intrinsic target specificity, adaptability and the immune response (Fisher et al., 2014; Mantovani et al., 2008).

The tumor microenvironment contains innate immune cells such as mast cells, neutrophils, basophils, eosinophils, cytotoxic cytokines related natural killer cells and IL-12 cytokine related macrophage and dendritic cells (Fig.3) (de Visser et al., 2006; Grivennikov et al., 2010). Macrophages have been identified as the key factors of neoplastic progression through the secretion of cytokines including tumor necrosis factor (TNF) and interleukin-1 (IL-1), and appropriately activated macrophages can derive cancer-destructive inflammatory responses towards an anti-tumor function (Kulbe et al., 2007; Szlosarek and Balkwill, 2003; Voronov et al., 2003; Yu et al., 2007; Zhu et al., 2006).

Indeed, several papers have reported that innate immune cells, especially natural killer cells, play a crucial role targeting tumor by their cytotoxic activity against cancer

(Caligiuri, 2008; Trinchieri, 1989; Vivier et al., 2008). Moreover, the crosstalk of natural killer cells with other immune cells such as dendritic cells is critical for anti-tumor response. And the anti-tumor response of natural killer cells by macrophages regulation via bidirectional interactions has been demonstrated (Cooper et al., 2004; Klezovich-Benard et al., 2012; Moretta, 2002; Nedvetzki et al., 2007; Tjwa et al., 2012; Walzer et al., 2005).

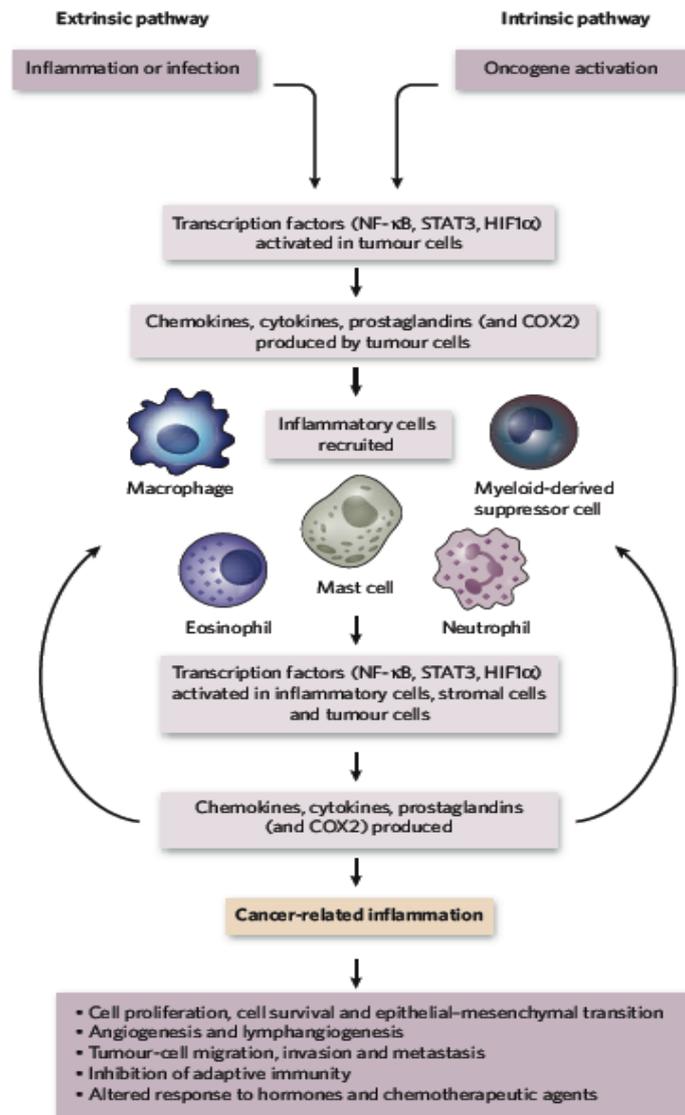


Figure 3. Pathways that connect inflammation and cancer

Figure 3. Pathways that connect inflammation and cancer

Cancer and inflammation are connected by two pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is activated by genetic events that cause neoplasia. These events include the activation of various types of oncogene by mutation, chromosomal rearrangement and the inactivation of tumor-suppressor genes. Cells that are transformed in this manner produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumors for which there is no underlying inflammatory condition. By contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites. The two pathways converge, and result in the activation of transcription factors, mainly nuclear factor- κ B (NF- κ B), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1 α (HIF1 α), in tumor cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines, as well as the production of cyclooxygenase 2 (COX2). The cytokines activate the same key transcription factors in inflammatory cells, stromal cells and tumor cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated (Mantovani et al., 2008).

Chapter I

**Growth arrest and forced
differentiation by
phosphodiesterase 4 type D
inhibitor in human primary
glioblastoma multiforme**

1.1 INTRODUCTION

Glioblastoma is the most prevalent and aggressive primary brain tumor in patients, with a median survival of less than a year after diagnosis due to its chemoresistance and radioresistance potential (Bao et al., 2006; Liu et al., 2006; Stupp et al., 2009). It has recently been accepted that undifferentiated cancer cells, called cancer stem cells (CSCs), in various tissues comprise only a small subpopulation of the tumor, but each single cell play a crucial role in the initiation and the progression of cancers which cause relapse and metastasis by giving rise to a new tumor (Beck and Blanpain, 2013). Regarding the biological characteristics of CSCs, recent studies have reported evidence that CSCs are similar to normal stem cells in self-renewal and multi-lineage differentiation potential, but they differ in their long-term proliferative capability.

Similar to tissue-specific stem cells (somatic or adult stem cells), there are no universal biological markers for CSCs. Nonetheless, the cell surface marker CD133 has been frequently used for characterization of tissue-specific stem cells. Over the last several years, CD133 expression has been detected extensively in various stem/progenitor cells, especially in the human neural systems, including the fetal brain, the post-mortem retina and embryonic stem cell-derived neural progenitors (Carter et al., 2009; Peh et al., 2009; Uchida et al., 2000). Moreover, CD133 has been most frequently applied in brain tumors as a putative CSCs biomarker (Singh et al., 2004). Recent studies have suggested that CD133 expressing cell subpopulation in GBM is thought to be a CSCs population (Bao et

al., 2006; Collins et al., 2005; Eramo et al., 2008; Singh and Dirks, 2007). This CD133 expressing subpopulation reveals its increased tumorigenic potential higher than CD133 negative subpopulation (Beier et al., 2007; Galli et al., 2004; Reya et al., 2001; Yuan et al., 2004). Furthermore, a reliable study demonstrated that targeting the CSCs subpopulation could be the key in GBM therapy (Lathia et al., 2010). There have been many attempts to develop therapeutic strategies targeting tumorigenic cell populations, but significant results have not yet been achieved.

Other than extermination of CSCs subpopulation, forced differentiation of tumor cells to limit their long-term proliferative potential is a new approach in the search for alternative cancer therapies. Piccirillo has demonstrated that bone morphogenetic protein 4 (BMP4) induces the neural differentiation in human primary GBM cells. He showed that BMP4 plays a role as a growth inhibitor on CD133-expressing GBM-derived cells in vitro and that BMP4 treatment interrupts tumorigenic activity in vivo (Piccirillo et al., 2006). Other studies have reported induced terminal differentiation using a chemical combination of retinoic acid and arsenic trioxide in leukemic cells and demonstrated that the depletion of the leukemic CSCs by using a chemical combination is involved with cAMP signaling pathway (de The and Chen, 2010; Guillemain et al., 2002; Nasr et al., 2008). Although multiple drugs were found to affect the phenotype of the tumor mass, the efficacy of these drugs was limited and did not affect the tumor-initiating cells.

A few studies have investigated whether phosphodiesterase 4 (PDE4) plays a pivotal role in tumor inhibition (Goldhoff et al., 2008; Moreno et al., 2006; Yang et al., 2007). PDE4 inhibitors have been frequently used to enhance cAMP/CREB signaling

pathway because PDE4 is well known for its role to reduce cAMP level in cells. The cAMP/CREB signaling pathway is participates in many biological functions and seems to play an important role in differentiation (Mayr and Montminy, 2001; Mayr et al., 2001). Moreover, the cAMP/CREB signaling pathway can induce the differentiation of human cancer cells and mesenchymal stem cells (Sato et al., 2006; Siddappa et al., 2008; Zhao et al., 2004). In cancer biology, it has been demonstrated that cAMP/CREB signaling pathway related genes play as oncogenes or oncogene inhibitors (Kirschner et al., 2000; Landis et al., 1989; Lania et al., 2001; Lucchi et al., 2011; Persani et al., 2000). Rolipram was recently used in a GBM research, due to its promoting cAMP signal transduction (Li et al., 2011). Rolipram is a specific PDE4 inhibitor which commonly known for memory enhancer (Otmakhov et al., 2004; Romano et al., 1996) owing to its role in up-regulating the level of cAMP and the phosphorylation of CREB (Park et al., 2012; Schneider, 1984). The Forskolin induces up-regulation of cAMP level and leads gap junctional intercellular communication enhancement, which is mostly inhibited in cancer cells, and to neuronal differentiation of SVG cells (Dowling-Warriner and Trosko, 2000). Although recent reports have suggested the pivotal role of cAMP/CREB signaling pathway in the differentiation of cancer cells, the cellular mechanism of forced differentiation process by cAMP and CREB is remains to be unraveled.

Several studies reported about the relationship between tumor suppression and neural differentiation in GBM. Nevertheless, the tumor-suppressive potential of PDE4D inhibitors have barely been studied. There are four PDE4 subfamilies (A to D), which are comprised of more than 20 different isoforms (Li et al., 2011) that regulate the cellular

cAMP level through their hydrolytic activity (Houslay and Adams, 2003; Marko et al., 2000). All the variants of PDE4 and their anti-cancer effects are not clearly known yet. However, the specific variant PDE4D inhibitor, CG500354, may provide a closer step forward to unveiling the therapeutic issues to target aggressive glioblastoma.

In this study, I demonstrate that the novel small molecule, CG500354, acts as a tumor-suppressor in human primary GBM by increasing p53, expression which subsequently compel growth arrest of GBM. I determined that CG500354 forces induction of the neural differentiation in GBM-derived cells through cAMP/CREB signaling pathway. In particular, I suggested that the well-known tumor suppressors, Rolipram and Forskolin, are also involved in cAMP/CREB signaling pathway as predicted and can induce the neural differentiation of GBM-derived cells. According to my data, CG500354, the novel small molecule, reveals its tumor suppressive potential and its specific mechanism which accompanied with p53 up-regulation and increased cAMP/CREB activity.

1.2 MATERIALS AND METHODS

1.2.1 Isolation of primary GBM cells

Following informed consent and in accordance with the appropriate institutional review boards, GBM was obtained from a patient undergoing surgery at the Samsung Medical Center (Seoul, Republic of Korea). Tumors were classified as GBM based on WHO criteria by examination of pathologists⁵¹. Within one hour of surgical resection, the tumor mass was mechanically and enzymatically dissociated into single cells. GBM was briefly maintained in NBE neurosphere culture medium: Neurobasal-A medium (Invitrogen, Carlsbad, CA, USA) supplemented with recombinant human basic fibroblast and epidermal growth factors (50 ng/ml each; Invitrogen), N2 and B27 supplements (Invitrogen), 2mM glutamine (Invitrogen), 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen). I used human primary cells from three different GBM origins: GBM559, GBM592 and GBM626.

1.2.2 Chemical

CG500354 (Patent No. 61/470884) was provided by Crystal Genomics, Inc. (Seongnam, Republic of Korea). Rolipram (C₁₆H₂₁NO₃; 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrroli-dinone) and Forskolin (C₂₂H₃₄O₇; 7b-acetoxy-8,13-epoxy-1a,6b,9a-trihydroxylabd-14-en-11-one) were purchased from Sigma Aldrich (Saint Louis, MO, USA). For the cell treatments, Rolipram and Forskolin were used at a concentration

of 10 μ M.

1.2.3 Clonogenic assays

Dissociated sphere cells (5×10^5) were cultured in ultra-low attachment dishes. The cells were treated with DMSO or CG500354 for 72 hours. The number of spheres with a diameter of $> 100 \mu$ m were counted randomly in triplicate cultures.

1.2.4 Magnetic-activated cell sorting

To isolate the CD133-positive GBM-derived cells from the DMSO- or CG500354-treated cells, I resuspended the treated cells with 300 μ l/108 buffer (PBS with 0.5% FBS) and added 50 μ l/108 of FcR Blocking Reagent and 50 μ l/108 CD133 MicroBeads (#130-050-801, Miltenyi Biotec) for 30 minutes at 6 $^{\circ}$ C to 12 $^{\circ}$ C. Then, I isolated CD133-positive cells using through Column and stained with anti-CD133/2-PE antibody (Miltenyi Biotec) to further analyze.

1.2.5 Flow cytometry analysis

GBM-derived cells were treated with DMSO or CG500354 for 72 hours and stained with an anti-CD133/2-PE antibody (#130-090-853, Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes. The CD133-stained GBM derived cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson).

1.2.6 cAMP measurement

GBM cells were treated with DMSO or CG500354 (3 μ M/ml) for 72 hours. The cAMP level was measured from the collected supernatants using a cyclic AMP EIA Kit (#581001, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. The cAMP values were calculated using a multifunction microplate reader (Tecan, San Jose, CA, USA).

1.2.7 siRNA inhibition study

To specifically inhibit PDE4D expression, siRNA-mediated gene knockdown studies were performed using commercial siRNAs targeting the PDE4D transcript (ON Target plus SMART pool, Dharmacon, Lafayette, CO, USA) along with a non-targeting siRNA (#D-001810-01, ON Target plus SMART pool, Dharmacon). The siRNA transfections were performed according to the manufacturer's instructions. Briefly, the cells were seeded at a concentration of 5×10^5 per well in 6-well plates. When the cells reached 60–70% confluence, a mixture of 50 nM siRNA and transfection reagent was added to the cell culture medium without antibiotics. The cells were incubated for 24 hours before evaluating mRNA expression by RT-PCR analysis or for 48 hours before evaluating protein expression by western blot analysis.

1.2.8 Quantitative real-time PCR

The relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method after normalization to the endogenous expression level of GAPDH.

1.2.9 Western blot analysis

For western blot analysis, the following primary antibodies were used: mouse monoclonal anti-GFAP (ab4648; Abcam, Cambridge, UK), mouse monoclonal anti-Tuj1 (MMS435; Covance, Princeton, NJ, USA), mouse monoclonal anti-P53 (2524; Cell Signaling Technology Inc., Boston, MA, USA), rabbit monoclonal anti-p-CREB (9198; Cell Signaling Technology Inc.), rabbit monoclonal anti-p-PKA (5661; Cell Signaling Technology Inc.) and mouse monoclonal anti-GAPDH (MAB374; Millipore, Billerica, MA, USA).

1.2.10 Immunocytochemistry

The primary antibodies used for immunocytochemistry were: mouse monoclonal anti-nestin (MAB5326; Millipore), mouse monoclonal anti-GFAP (ab4648; Abcam), mouse monoclonal anti-Tuj1 (MAB1195; R&D Systems, Minneapolis, MN, USA) and mouse monoclonal anti-P53 (2524; Cell Signaling Technology Inc.). Both fluorescent secondary antibody conjugates, goat anti-mouse Alexa Fluor 488 (Invitrogen) and goat anti-rabbit Alexa Fluor 488 (Invitrogen), were used at a 151000 dilution. DAPI was used for counterstaining. The coverslips were mounted with fluorescent mounting solution (DAKO, Carpinteria, CA, USA) and observed by confocal microscopy (Eclipse TE200, Nikon, Japan).

1.2.11 Animal experiment

NOD-SCID mice were purchased from Jackson Laboratories (Bar Harbor, ME,

USA). All experiments were performed in accordance with the guidelines and regulation, which were approved by the Institute of Laboratory Animals Resources (SNU-101013-5, Seoul National University, Republic of Korea). Four-week-old female mice were subcutaneously injected with 1×10^7 GBM-derived cells, which were suspended in 0.1 ml PBS. When tumors appeared, the mice were randomly divided into two groups and treated daily with intraperitoneal injections of 3 mg/kg CG500354 (n = 4) or DMSO as the vehicle control (n = 3) for 10 days. The mice were sacrificed, and the tumors were separated from the host for histological evaluation.

1.2.12 Statistical analysis

All experiments were conducted at least in triplicate, and the results are expressed as the mean \pm SD. The statistical analyses were conducted using analysis of variance (ANOVA) followed by a Duncan's multiple range test or Student's t-test. The following P values were considered significant: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

1.3 RESULTS

1.3.1 CG500354 induces differentiation in human primary GBM-derived neurospheres without cytotoxic effects

I first determined the effects of a novel small molecule, CG500354, on the oncogenic phenotype of human primary GBM-derived cells. CG500354 has been designed, synthesized and evaluated by Crystal Genomics, Inc. using its structure-based drug discovery platform which employs the integrated technologies of the SPSTM (Soluble Protein Solution), technology for obtaining soluble forms of disease related proteins that are usually insoluble when overexpressed in heterologous systems, SCPTM (Structural Chemo Proteomics), technology for rapidly generating novel leads using the 3-D structure information of the target proteins, and SDFTM (Structural-based Drug Factory), technology to optimize novel leads to drug candidate compounds using the 3-D structure information of the complexes of target proteins and inhibitors. The synthetic compound, CG500354, is an inhibitor of cAMP-specific 3'.5'-cyclic phosphodiesterase 4D (PDE4D), which plays a role in cAMP homeostasis by hydrolyzing the second messenger cAMP (Fig. 1A). Human primary cells derived from three different GBM origins, GBM559, GBM592 and GBM626, were grown as neurospheres under floating cell culture conditions (Fig. 1B). A very small portion of human GBM cells expresses CD133, which is a putative biomarker of neural stem cells and CSCs. It is known that this CD133 expressing subpopulation plays a crucial role in the proliferation and survival of GBM. To classify human primary GBM

samples, I isolated the CD133 subpopulation from the GBM-derived cells using magnetic-activated cell sorting (MACS). Then, I performed flow cytometry to detect the CD133 expressing GBM-derived cells and verified the present of CD133-expressing subpopulation (Fig. 1C). In a xenograft mouse model the GBM-derived cells were able to form a glioblastoma. This formed tumor showed two typical histopathological features of GBM: pseudopalisading necrosis and endothelial proliferation (Fig. 1D).

To identify the effects of CG500354 *in vitro*, I performed a clonogenic assay with treatment of CG500354 or dimethyl sulfoxide (DMSO) as a vehicle control for 72 hours on GBM-derived neurospheres cultured in non-adherent culture conditions. In the result of clonogenic assay, the CG500354-treated neurospheres revealed the smaller neurosphere numbers compare to the DMSO-treated neurospheres (Fig. 1E). While I seeded neurospheres on the PLO/FN-coated dishes for immunocytochemistry, I could observed that most CG500354-treated neurospheres turn into adherent and differentiated state, but many DMSO-treated neurospheres still remain as a sphere (Fig. 1F). However, in viability assay, number of GBM cells was not prominently changed in response to 72hours of CG500354 treatment (Fig. 1G). These results suggest that the small molecule, CG500354, induces a dramatic change in the morphology of human GBM-derived neurospheres without cellular cytotoxicity.

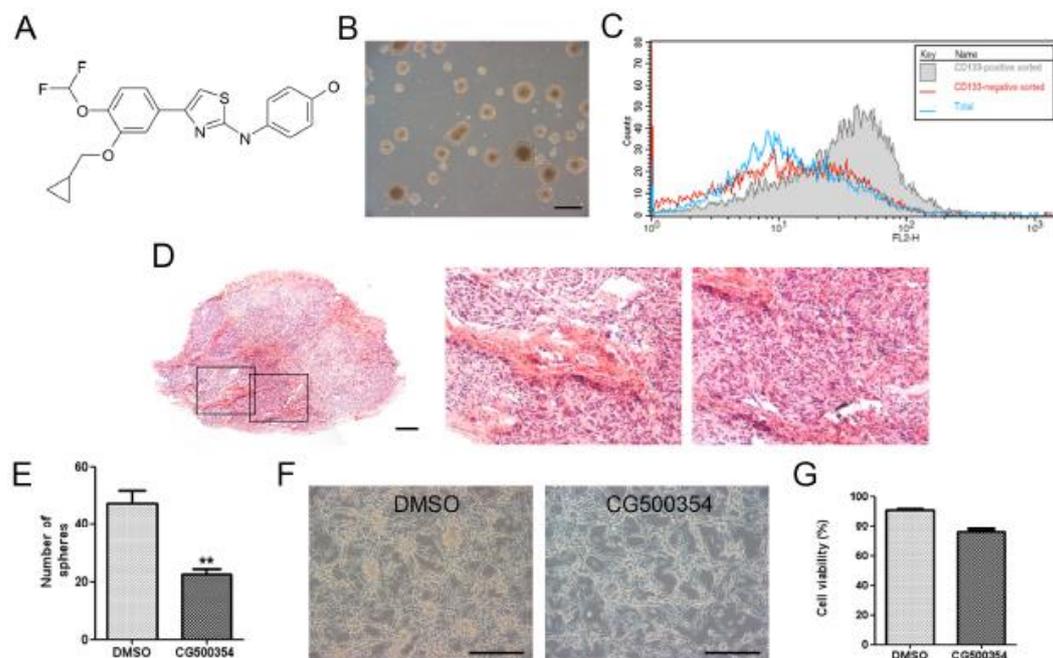


Figure 1. Identification of human primary GBM-derived cells.

(A) Chemical structure of the novel small molecule CG500354. **(B)** Phase-contrast morphological image of GBM-derived spheres in non-adherent culture conditions. Scale bar = 1 mm. **(C)** Characterization of CD133 expressing GBM-derived cells after magnetic-activated cell sorting by using flow cytometry analysis. **(D)** Characterization of pseudopalisading necrosis (middle) and endothelial proliferation (right) after hematoxylin and eosin staining in glioblastoma tumor which isolated from in a xenograft mouse model. Scale bar = 400 μ m. **(E)** The number of neurospheres significantly decreased by 72hours of CG500354 treatment in a clonogenic assay. **(F)** Representative phase-contrast images of GBM-derived cells after DMSO or CG500354 treatment. Scale bar = 1 mm. **(G)** A trypan

blue exclusion test was performed to calculate the number of viable cells compared to the total cell number in the CG500354 (3 μ M) or DMSO-treated GBM-derived cell population. DMSO was used as a vehicle control.

1.3.2 CG500354 induces growth arrest of GBM-derived cell populations by up-regulating p53

I performed a cell cycle analysis to identify the growth-suppressive potential of CG500345 in human primary GBM-derived neurospheres. In the presence of CG500354, the G1 fraction of the total neurosphere cells was increased approximately 10% while the G2 fraction was not detected (Fig. 2A). Moreover, I performed a quantitative RT-PCR analysis for additional evidence of the cell cycle arrest (Table. 1). Cell cycle regulators compose a complex regulatory system and include cyclins and cyclin-dependent kinases (Cdks). Cdks are inactivated unless bound to phase-specific cyclins. Here, I assessed the mRNA expression of cyclin A1 for S phase, cyclin B1 for M phase and cyclin D1 and D3 for G1 phase were significantly decreased in the presence of CG500354 (Fig. 2B). I further analyzed the gene expression level of two Cdk inhibitor proteins, p21 and p27, and found that both were significantly up-regulated (1.95-fold and 1.35-fold, respectively) in CG500354-treated GBM-derived neurospheres (Fig. 2C). These results indicate that CG500354 induces growth arrest in GBM derived neurospheres by inhibiting cell cycle regulators.

The tumor suppressor protein p53 is a transcription factor that has crucial effects on cell cycle arrest, DNA repair, apoptosis, senescence and angiogenesis (Brown et al., 2009; Riley et al., 2008). Thus, I analyzed p53 and its pro-apoptotic targets following CG500354 treatment. In our results, CG500354 treatment with 1 μ M and 3 μ M increased the protein expression level of p53 in the GBM-derived neurospheres (Fig. 2D). In immunocytochemistry, CG500354 treatment increased the number of p53-expressing cells

from 55.2% to 86.3% by 1 μ M CG500354 and to 93.1% by 3 μ M CG500354 (Fig. 2E–F). Western blot analysis shows increased expression level of pro-apoptotic targets, BAK and BAX (Fig. 3). These data suggested that the growth arrest was triggered by p53 and its downstream targets, such as p21, p27 and pro-apoptotic targets, whereas the forced neural differentiation was induced by cAMP/CREB signaling pathway.

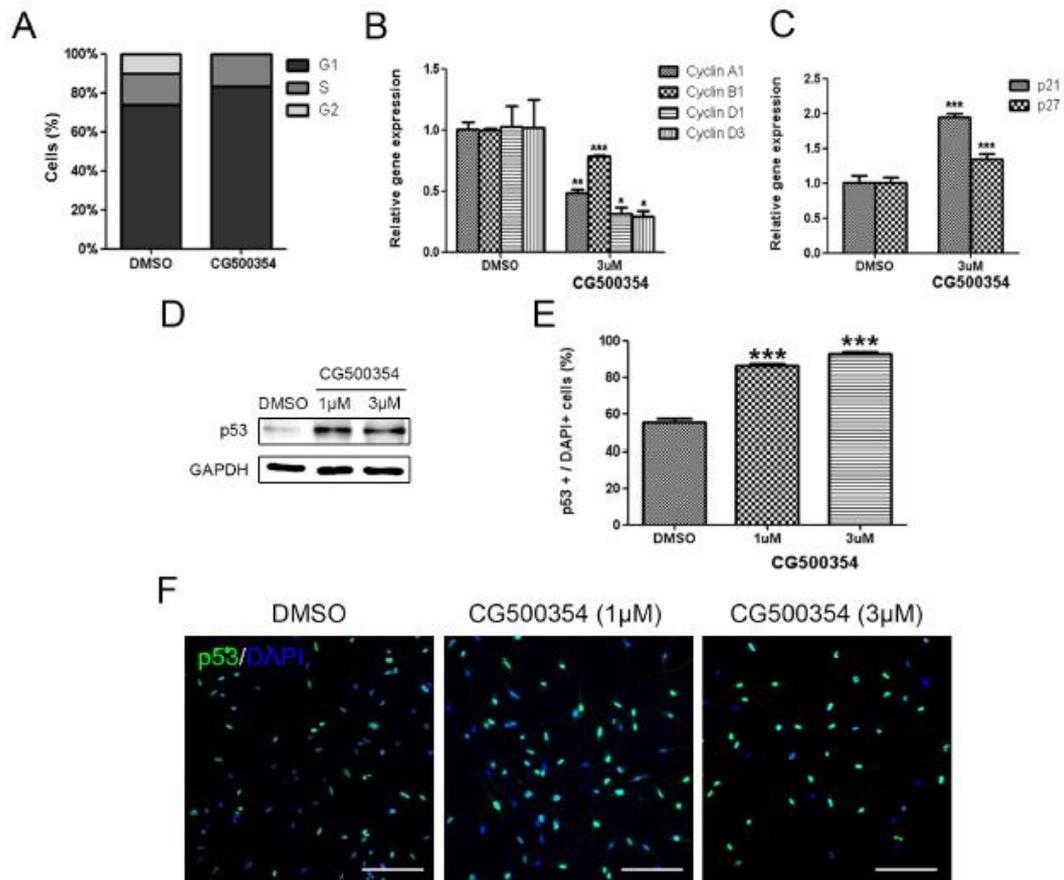


Figure 2. Growth arrest of human primary GBM-derived cells through p53 overexpression.

(A) Increased percent of the G1 phase was detected after CG500354 treatment in cell cycle analysis. (B–C) In the presence of CG500354, Cdk inhibitor proteins, p21 and p27 mRNA levels were significantly increased while the cell cycle regulators cyclin A1, B1, D1 and D3 were significantly down-regulated in quantitative RT-PCR analysis. (D) Significantly increased protein expression level of p53 was detected by western blot analysis in both two

different concentration (1 μ M or 3 μ M) of CG500354. (E–F) GBM-derived cells treated with both concentrations (1 μ M and 3 μ M) of CG500354 were stained with a p53 antibody. The number of p53-expressing cells (green) were significantly increased after in both concentrations (1 μ M and 3 μ M). Nuclei (blue) were counterstained with DAPI. Scale bar = 0.5 mm.

Table 1. Primer list for quantitative RT-PCR analysis

Gene	Forward	Reverse	cDNA product(s) (bp)	gDNA product (bp)
Nestin	ACCTCAAGATGTCCC TCAGC	GAGCAAAGATCCAA GACGCC	176 and 89	2112
CCNA1	GTCACTTGGGATGG AGACCG	GCATTGCTTCAGACT CCACG	143	449bp
CCNB1	GCCACGAACAGGCC AATAAG	CGCCTGCCATGTTGA TCTTC	250	874bp
CCND1	CCCTCGGTGTCCTAC TTCAA	TGGAGGGCGGATTG GAAATG	487	X
CCND3	AGACTGGCTCTGTTC GGATG	GCAAGACAGGTAGC GATCCA	238	X
p21	AGTCAGTTCCTTGTG GAGCC	CATTAGCGCATCACA GTCGC	184	X
p27	ACCTGCAACCGACG ATTCTT	GTCCATTCCATGAAG TCAGCG	252	X
p53	TGTGACTTGCACGTA CTCCC	ACCATCGCTATCTGA GCAGC	199	X
GAPDH	CATGAGAAGTATGA CAACAGCCT	AGTCCTTCCACGATA CCAAAGT	185	X

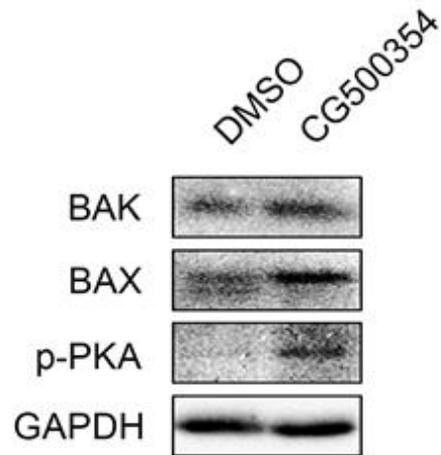


Figure 3. Expression level of pro-apoptotic targets.

Protein expression level of two apoptotic markers and phosphorylated PKA in GBM-derived cells were increased after a CG500354 treatment.

1.3.3 CG500354 induces the neural differentiation of GBM-derived cells

To verify the neural differentiation potential of CG500354 in human primary GBM-derived cells, 2×10^4 cells were seeded on PLO/FN-coated coverslips and treated with two different concentration (1 μ M or 3 μ M) of CG500354. After 72 hours of CG500354 treatment, the cells exhibited the neuronal morphology thus I investigated the expression of the neural progenitor cell marker nestin, the neuronal marker Tuj1 and the astrocyte marker GFAP by using immunocytochemistry (Fig. 4A). In the vehicle control, 92.4% of the cells expressed nestin, and this percentage was significantly reduced to 20.3% after 3 μ M CG500354 treatment, whereas 1 μ M CG500354 did not affect nestin expression (Fig. 4B). Analysis of nestin expression at the mRNA level showed identical results as predicted (Fig. 4C). In opposition to nestin, the number of GFAP- and Tuj1-expressing cells was significantly increased upon CG500354 treatment. In comparison to the vehicle control, the cell numbers rose up to 6-fold in GFAP and 5-fold in Tuj1 with 3 μ M CG500354 (21.4% and 79.6%, respectively). In addition, western blot analysis showed similar expressing pattern of GFAP and Tuj1 with immunocytochemistry (Fig. 4D). I observed that the neural progenitor/precursor marker expression was changed in a dose dependent manner. From the above, the concentration of 3 μ M CG500354 was most efficient in the neural differentiation of human GBM-derived cells. Altogether, these results indicate that CG500354 induces the neural differentiation with GFAP and Tuj1 up-regulations.

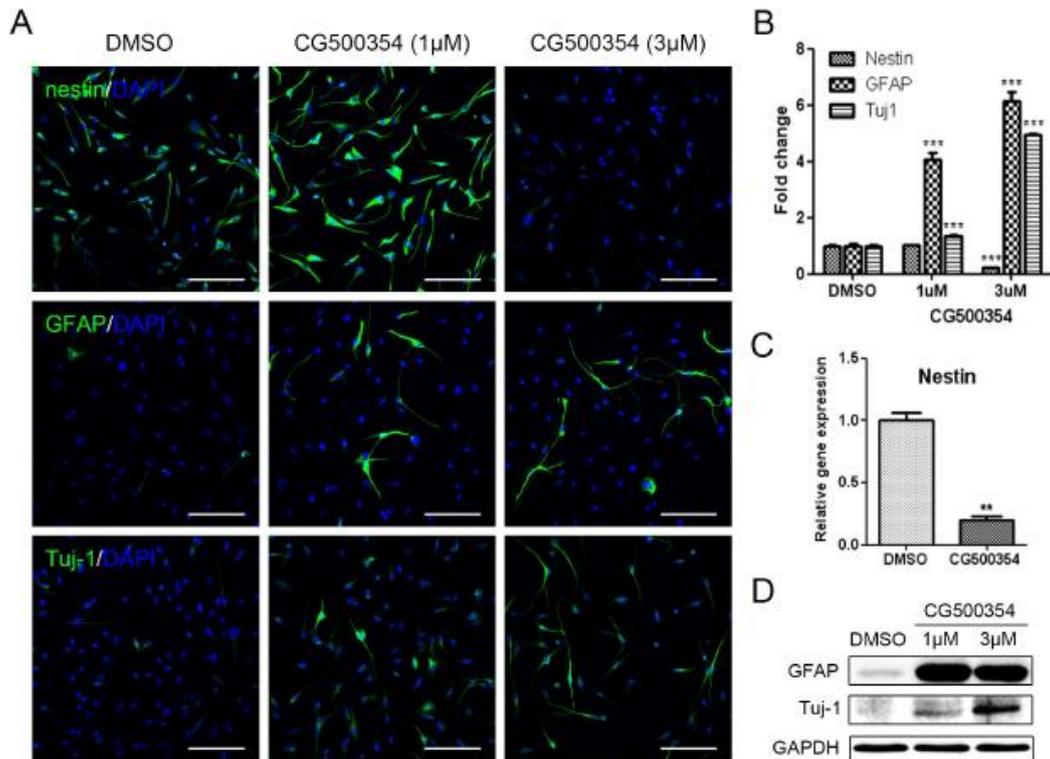


Figure 4. Induction of neural differentiation by CG500354 treatment.

(A–B) Representative immunochemical images of primary GBM-derived cells show that CG500354 treatment decreased the number of nestin-positive neural progenitor cells and increased the number of GFAP- and Tuj1-positive neural subtypes. The effect of CG500354 on the neural differentiation of GBM was more effective in concentration of 3 μ M than 1 μ M CG500354. Nuclei (blue) were counterstained with DAPI. Scale bar = 0.5 mm. (C) Quantitative RT-PCR analysis shows that the relative expression level of nestin was significantly reduced by 3 μ M CG500354. (D) Western blot analysis shows that the expression of the GFAP and Tuj1 proteins was up-regulated by CG500354 treatment.

1.3.4 CG500354 leads GBM-derived cells to growth arrest by accelerating the cAMP/CREB signaling pathway

As previously described, CG500354 is an inhibitor of PDE4D, which regulates the cAMP signaling. In the cAMP/CREB signaling pathway, the accumulation of cAMP induces the diffusion of a subunit of cAMP dependent phosphorylated protein kinase A (PKA) into the nucleus and activates cAMP response element-binding protein (CREB). In several studies, CREB is described to stimulate the differentiation of human cancer cells and mesenchymal stem cells (Sato et al., 2006; Siddappa et al., 2008). Here, I examined the CG500354-mediated activation of the cAMP/CREB signaling pathway. After CG500354 treatment, the supernatants from the cell culture of GBM cells were harvested and measured the secreted cAMP concentration. These measurements revealed an approximately 4.5-fold increase in the level of cAMP secretion in the CG500354-treated GBM-derived cells compared to the control cells (Fig. 5A). I then performed western blot analysis of the major downstream targets PKA and CREB to verify the activation of the cAMP signaling pathway. CG500354 treatment effectively increased the expression of both phosphorylated PKA and CREB, and this up-regulation was detected from both CG500354 doses (Fig. 5B).

To compare the potential of CG500354 in GBM with two known mimetic substances, I treated GBM-derived cells with the cAMP regulator Forskolin and the PDE4 inhibitor Rolipram. Then, I confirmed both mimetic substances increased the level of phosphorylated CREB as CG500354 (Fig. 5C). Quantification of the western blot analysis showed that the levels of phosphorylated CREB in the Forskolin-treated cells were

approximately 11-fold higher than that in the control cells, but not as much as CG500354. In addition, I performed the direct suppression of PDE4D expression using a siRNA, targeting the PDE4D gene (si-PDE4D). This si-PDE4D reduced 73.6% of the PDE4D expression and increased the level of phosphorylated CREB in the human primary GBM-derived cells as expected (Fig. 5C and Fig. 6).

To explore whether these two mimetic substances can also induce growth arrest like CG500354, I treated GBM-derived cells with CG500354, Forskolin or Rolipram for 72 hours, and the treated GBM-derived cells were analyzed using immunocytochemistry (Fig. 5E–F). Similar to the CG500354-treated cells, the Forskolin- and Rolipram-treated cells showed significant increases in p53 protein expression. The percentages of p53-expressing cells in both of the groups treated with mimetic substances were as high as that observed in the group treated with CG500354. Moreover, treatment with Forskolin and Rolipram induced the gene expression of the p53 downstream targets p21 and p27 (Fig. 5D). The Rolipram significantly increased the expression of both p21 and p27, which inhibit cell cycle progression. Therefore, CG500354 and its mimetic substances Forskolin and Rolipram are able to induce growth arrest in GBM-derived cells. From the analyses of cAMP signaling and cell cycle regulators, I concluded that CG500354 appeared more effective than both mimetic substances and it takes a novel therapeutic potential to handle the GBM growth arrest.

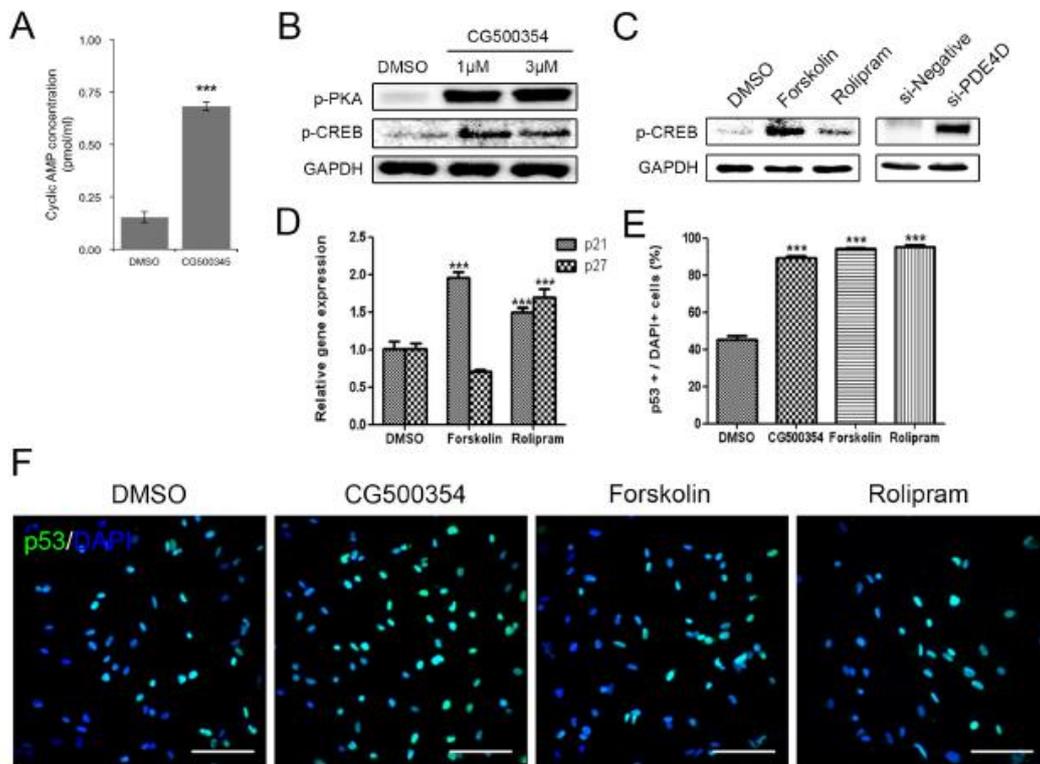


Figure 5. Activation of cAMP/CREB signaling pathway by CG500354 and mimetic substances.

(A) Measurement of the extracellular cAMP level was performed with a Cyclic AMP EIA Kit. The supernatant from the CG500354-treated GBM cells showed a significantly higher level of cAMP than that from the DMSO-treated GBM cells. (B) Western blot analysis shows that the expression of phosphorylated PKA and CREB was strongly induced by CG500354 treatment. (C) Both mimetic substances (Forskolin and Rolipram) and si-PDE4D also increased the expression of phosphorylated CREB. (D) Quantitative RT-PCR analysis reveals that both mimetic substances induced p21 expression and only Rolipram also increased p27 expression. (E–F) GBM-derived cells treated with CG500354,

Forskolin or Rolipram were stained with a p53 antibody. The numbers of p53-expressing cells (green) in the three groups were all equivalently high. Nuclei (blue) were counterstained with DAPI. Scale bar = 0.5 mm.

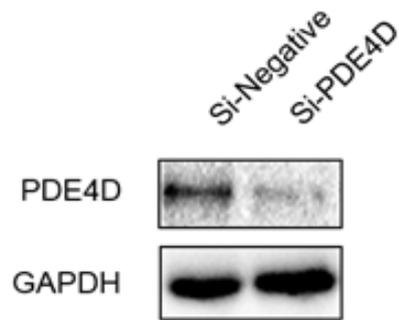


Figure 6. Knockdown of PDE4D expression using a PDE4D-siRNA.

Direct suppression of PDE4D protein expression in GBM-derived cells was examined after PDE4D-siRNA treatment by western blot analysis.

1.3.5 Mimetic substances and si-PDE4D, mimic the effect of CG500354 on the neural differentiation of GBM-derived cells

I next investigated the effect of these mimetic substances on the neural differentiation in human primary GBM-derived cells. After Forskolin and Rolipram treatment, the neural differentiation markers GFAP and Tuj1 showed the up-regulation in the western blot analysis (Fig. 7A). I further performed the immunocytochemistry to analyze the nestin, GFAP and Tuj1 expressions (Fig. 7B–C). Similar to CG500354, Forskolin- and Rolipram-treatment cells were detected 8 to 10-fold more GFAP-expressing cells and 5-fold more Tuj1-expressing cells in comparison to the vehicle control. Unlike CG500354, the number of nestin-expressing cells did not change after treatments of two mimetic substances. These results suggest that Forskolin and Rolipram induce the differentiation of GBM derived cells but do not deplete the cell population remaining in the neural progenitor state.

To further investigate the neural differentiation phenomena induced by CG500354, I performed gene silencing of PED4D using siRNA and it induced the overexpression of GFAP and Tuj1 in the GBM-derived cells as expected. The protein expression levels of GFAP and Tuj1 were significantly up-regulated in the si-PDE4D transfected GBM-derived cells (Fig. 7D). Moreover, the immunocytochemical analysis showed that the decrease of nestin-expression and increase of GFAP- and Tuj1-expression (Fig. 7E–F). Both our immunocytochemistry and western blot analyses revealed that si-PDE4D affects the expression of neural differentiation markers similar to CG500354. From the above, I could indicate that novel CG500354 have shown higher potential in cell cycle

regulation than Rolipram while activating cAMP signaling pathway as Forskolin. Therefore, our data on the mimetic substances and si-PDE4D provide direct evidence that CG500354 works as a multi-controller that induces neural differentiation and growth arrest in human primary GBM-derived cells.

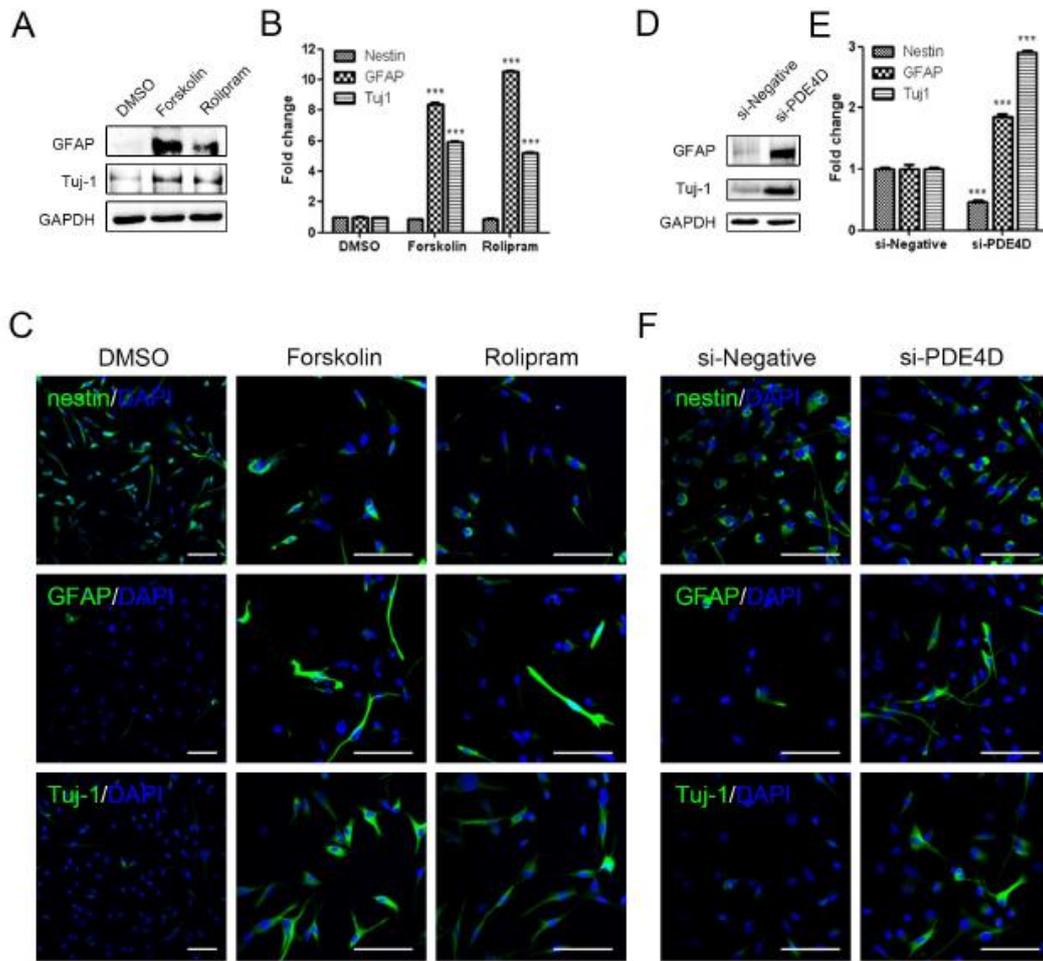


Figure 7. Induction of neural differentiation of human GBM by mimetic substances and si-PDE4D.

(A), (D) Western blot analysis shows that the expression of the neural subtype markers GFAP and Tuj1 was up-regulated upon Forskolin, Rolipram and si-PDE4D treatment. (B–C), (E–F) Representative immunochemical images of primary GBM-derived cells reveal that Forskolin, Rolipram and si-PDE4D induced an increase in the number of the GFAP-

and Tuj1-positive cells. Upon Forskolin or Rolipram treatment, the nestin-expressing cell population did not decrease (**B**), whereas this population was reduced by si-PDE4D as expected (**E**). Nuclei (blue) were counterstained with DAPI. Scale bar = 0.5 mm.

1.3.6 In vivo GBM-derived cells from tumor were induced to neural differentiation by CG500354 injection

To validate the in vivo effects of CG500354, I performed subcutaneous xenotransplantation of GBM-derived cells into NOD/SCID mice. After GBM tumor formation, I treated mice with CG500354 or DMSO via intraperitoneal injection for 10 days. Then, I sacrificed the mice and isolated GBM tumors from the host for hematoxylin and eosin staining (Fig. 8A). These GBM tumors were characterized with pseudopalisading necrosis, endothelial proliferation and irregular nuclear contours. Most part of the tumor showed a small nuclear size and 29.1% of this part appeared to be Tuj1-positive (Fig. 8B). But, the other part of the tumor showed a large nuclear size and 11.4% of this part appeared to be GFAP-positive by immunohistochemistry (Fig. 8C). These results indicated that approximately 40% of the GBM tumor was induced to differentiate into neural subtypes by CG500354 intraperitoneal injection.

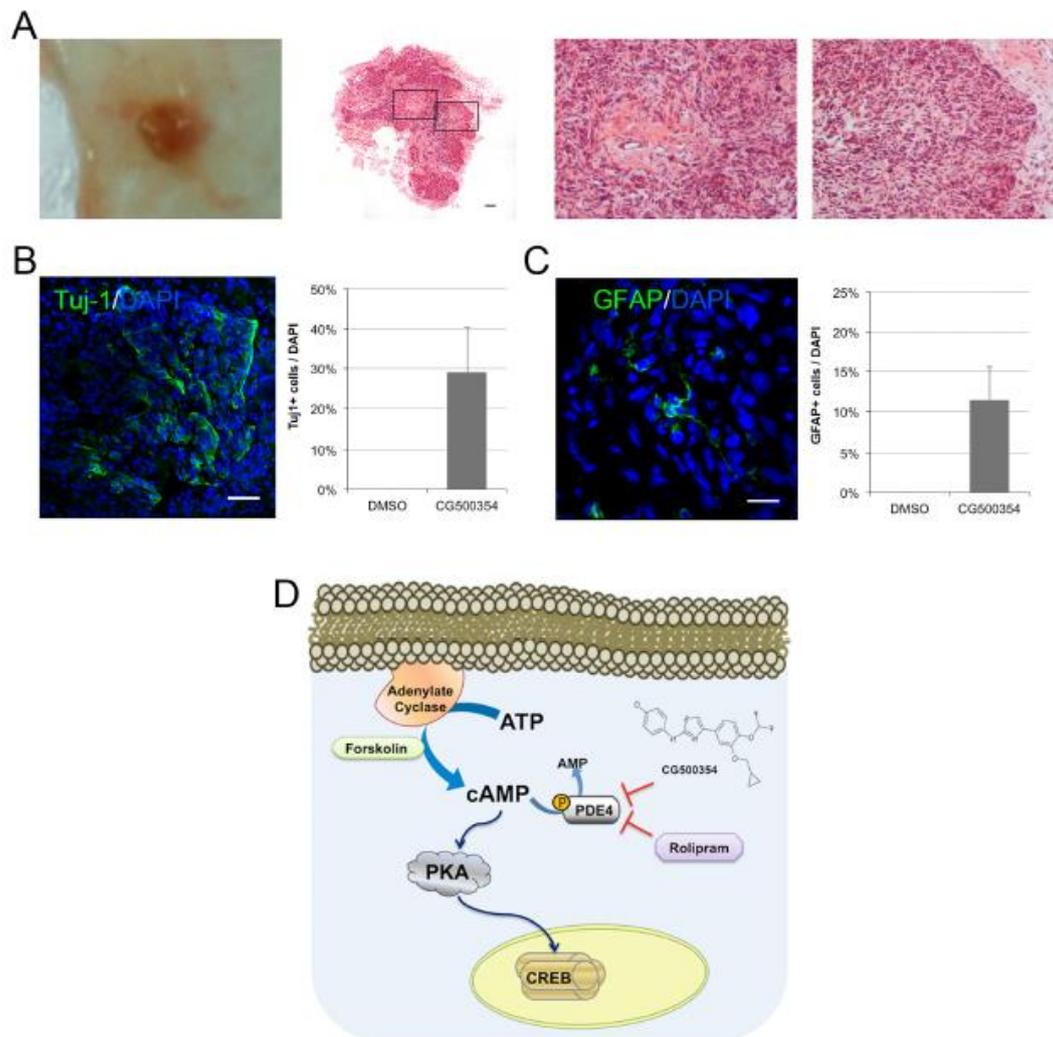


Figure 8. In vivo neural differentiation of human primary GBM-derived cells after xenotransplantation.

(A) After GBM tumor formation, tumors were isolated and stained with hematoxylin and eosin. A composited image (second from left) reveals an entire section of a tumor and two high-resolution images indicates pseudopalisading necrosis (second from right) and endothelial proliferation (right). (B–C) Representative immunochemical images of sections

from GBM-derived tumors show that the cells inside of the tumors were forced to differentiate into Tuj1- and GFAP-expressing neural subtypes. **(D)** Schematic diagram of the mechanism of CG500354-triggered cAMP/CREB signaling pathway. Likewise, both of the mimetic substances Forskolin and Rolipram are involved in this signal transduction pathway.

1.4 DISCUSSION

In this study, I investigated the dual effects of CG500354 as a PDE4D inhibitor on human primary GBM. First, I showed that CG500354 induces growth arrest and attenuates stemness in GBM derived cells. In human GBM cells, this small molecule regulates the expression of the tumor suppressor p53 and its downstream target p21 as well as p27. The increased expression of both Cdk inhibitors led to the decreased expression of a number of phase-specific cyclins and a reduction in the clonogenic potential of the GBM-derived cells. Second, CG500354 accelerated the neural differentiation, which were expressed Tuj-1 and GFAP in GBM-derived cells. Our findings suggest that the cAMP/CREB signaling pathway is involved in this neural differentiation via the phosphorylation of PKA and CREB following the CG500354-mediated up-regulation of cAMP.

In the preliminary study, I have done all experiments with three different GBM origins, including GBM559, GBM592 and GBM626, to analyze CG500354 effects. Those three GBM origin-derived cell types have shown similar results. Coincidentally, a quantitative RT-PCR analysis showed reduced gene expressions of SOX2 and nestin, and an increased gene expression of p53 in GBM559-, GBM592- and GBM626-derived cells. I have representatively shown the results of GBM559 in this study.

Recent studies have demonstrated that the regulation of GBM CSCs is approached from inhibition of the DNA-damage checkpoint kinases CHK1 and CHK2 using small molecules (Bao et al., 2006). Using this strategy indicated an improved radiotherapy for GBM CSCs. Another alternative strategy of GBM treatment is the induction of a

differentiated state of CSCs. A reliable report has demonstrated that GBM CSCs could be differentiated into endothelial cells, which could suppress the vasculature development (Ricci-Vitiani et al., 2010). However, the tumor-derived endothelial cells exist at a small portion in GBM tumor mass and its clinical relevance is questionable (Rodriguez et al., 2012). Zheng and colleagues demonstrated that the p53 together with PTEN could regulate the Myc expression in human primary GBM (Zheng et al., 2008). These GBM-derived cells after Myc suppression induced the down-regulation of nestin expression while they differentiated into Tuj1- and GFAP-expressing neural cell types. In this study, I assessed the GBM cell population that remained in the neural progenitor state, in which the nestin-expressing GBM CSCs reside. Interestingly, this cell population was diminished by CG500354 treatment but was not altered by treatment with mimetic substances (Fig. 4B and Fig. 7B). These results show that CG500354 plays a better role in human primary GBM and functions even better than Forskolin and Rolipram with respect to its potential anti-cancer effects.

From our findings on the mechanism of action of CG500354, I argue that p53 is implicated in the phenotype changes of the GBM derived cells. Several studies have commonly identified mutations of tumor suppressor genes, such as p53, PTEN and RB, in brain tumor (Chow et al., 2011; Verhaak et al., 2010). As shown in Figures 2F and 3B, I determined that the level of p53 expression was significantly increased after CG500354 treatment and was followed by differentiation into neural subtypes. I used QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) software to analyze molecular interactions between PDE4D and p53. Based on IPA,

representative regulatory networks were constructed that PDE4D directly activates PKA and cAMP, and indirectly activates p21 and p27 as well as p53. Another reason for the dual effects of CG500354 is that the PDE4D inhibitor CG500354 could affect the cAMP/CREB signaling pathway, which is widely known as a signal dependent gene regulatory pathway that is involved in many biological functions, including the cell cycle, cell survival and cell differentiation (Mayr and Montminy, 2001; Mayr et al., 2001). In the cAMP-dependent signal transduction pathway, PDE4D plays a suppressive role, as it causes a decrease in the level of cAMP. In a recent report on the role of cAMP, PDE4 was indicated as the most important target for the induction of apoptosis and differentiation in leukemia cells (Copsel et al., 2011; Karin, 1994). In another study, PDE4D was demonstrated to be abundantly expressed in glioblastoma cells and its inhibitor Rolipram was found to induce tumor regression (Goldhoff et al., 2008). Likewise, our data in Figure 4 reveals that CG500354, Forskolin and Rolipram could regulate the level of cAMP and its downstream target CREB. Together with data described above, our results indicate that CG500354 up-regulates the cAMP/CREB signaling pathway activity and induces the differentiation of human primary GBM.

In conclusion, I have shown that the protein level of p53 and the extracellular level of cAMP are significantly increased upon CG500354 treatment. I further indicated that CG500354 can alter the phenotypes of human primary GBM cells from an oncogenic state into a less oncogenic and more differentiated state. During these tumor-suppressive events, p21- and p27-mediated growth arrest and CREB-mediated neural differentiation also occurred. Moreover, the effects of CG500354 on the neural differentiation of the cells were

more significant with respect to its reduction of the nestin-expressing population than the mimetic substances Forskolin and Rolipram. Therefore, the novel small molecule CG500354, which targets PDE4D, might be important in the development of new drugs for human GBM.

Chapter II

Mica Nanoparticle Eliminates the Human Breast Carcinoma Cells by Regulating the Interaction of Tumor with its Immune Microenvironment

2.1 INTRODUCTION

Breast cancer is one of the most common cancers developed in women, with high occurrence and fatality rates. Annually, approximately 1.38 million women worldwide are diagnosed with this disease, which is the second leading cause of cancer deaths among women in the US. (Jemal et al., 2011). The surgery, chemotherapy, radiation therapy and immunotherapy are the most prevalent types of cancer therapy (Alama et al., 2012; Blagosklonny, 2003; Dey et al., 2008). However, the primary treatment is based on surgery or chemotherapy, which still have the issues of enhancing metastasis, systemic toxicity and drug resistance. These therapeutic limitations led researchers to develop targeted cancer therapies. Drugs or other natural compounds have been developed as targeted therapy for cancer to suppress the proliferation and metastasis. Therefore, several natural compounds such as plant extracts, minerals, vitamins or the combination of these compounds, have been suggested as alternative anti-tumor medicines (Feldman et al., 2014; Liu et al., 2013; Vekariya et al., 2012).

Mica has been demonstrated to have the anti-tumor and the immunostimulatory potential. Cho and colleagues reported that mica is revealed as a chemopreventive agent against colorectal cancers (Cho et al., 2013). Moreover, mica nanoparticles have been used as feed supplements in domestic animals to enhance immune activity owing to its immune stimulation responses against virus infection (Jung et al., 2013; Jung et al., 2010; Jung et al., 2015a). Recently, Jung et al. reported macrophage activation through up-regulation of lysosome and phagosome pathway which are caused by mica nanoparticles. (Jung et al.,

2015b). Although the immunostimulatory ability can be efficiently used to suppress tumor, most studies using mica have investigated only the anti-tumor or the immunostimulatory effects separately. None have tried to incorporate these two effects by elucidating mechanistic action of mica on immune cells constituting tumor microenvironment. In addition, till now, a study regarding the anti-tumor effect of mica has been performed against only one type of cancer, even though there are more than 100 types of cancers have been isolated and identified including breast cancer cells (Brody, 2011).

Natural Killer (NK) cells are innate immune cells that play a pivotal role in anti-tumor response by activating strong cytotoxicity against cancer cells (Trinchieri, 1989). Upon activation, NK cells eliminate target cells by secreting cytolytic enzymes or cytokines including interferon (IFN)- γ (Caligiuri, 2008; Vivier et al., 2008). Moreover, the crosstalk of NK cells with other immune cells is important for anti-tumor responses. For example, DCs can activate resting NK cells, which in turn, induce DC maturation (Cooper et al., 2004; Moretta, 2002; Walzer et al., 2005). Recently, it has been demonstrated that macrophages also regulate the anti-tumor responses of NK cells via bidirectional interactions (Klezovich-Benard et al., 2012; Nedvetzki et al., 2007; Tjwa et al., 2012). Bellora *et al.* reported that immunostimulatory type of macrophages (M1) induces strong activation of resting NK cells and the polarization of macrophages toward M1 phenotype can restore immunomodulatory type of macrophages (M2), the general phenotype in cancer tissues (Bellora et al., 2010). These findings indicate that the crosstalk between NK cells and other immune cells might be an important target of mica to promote anti-tumor immunity. Therefore, the present study was designed to investigate the anti-tumor effect of

STB-HO (mica nanoparticles) against MCF-7, the human breast carcinoma cell line. Furthermore, in an attempt to verify the underlying mechanisms, I explored the direct effects of STB-HO on MCF-7 as well as the indirect, immunity-mediated effects.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

LPS was purchased from Invivogen (San Diego, CA). IFN- γ was purchased from PeproTech (Rocky Hill, NJ) and IL-12 from R&D Systems (Minneapolis, MN).

2.2.2 MCF-7 xenograft model

Athymic nude mice (female, 5wk old) were obtained from SLC (Hamamatsu, Japan) and mice were group-housed under specific pathogen-free conditions in the animal facility of Seoul National University. All experiments were performed in accordance with the guidelines and regulation, which were approved by the Institute of Laboratory Animals Resources (SNU-140103-5, Seoul National University, South Korea). 2×10^6 MCF-7 cells mixed with 0.1 mL matrigel (Corning, Corning, NY) were subcutaneously injected into the right flank of athymic nude mice. After one week of stabilization period, the mice were divided into three groups and orally administrated with 35 mg/kg STB-HO (n = 8) or 70 mg/kg STB-HO (n = 7) or normal saline as the positive control (n = 7) daily for 12 weeks. Tumor size was measured twice a week using a caliper. Tumor volume (V) was calculated as the formula: $V \text{ (mm}^3\text{)} = \text{length} \times \text{width}^2 / 2$. After sacrifice, tumors were isolated for size measurement, histological evaluation and signaling analysis on protein level.

2.2.3 Histological evaluation

Tumor samples were collected, fixed in 10% formalin, subjected to consecutive steps of alcohol-xylene changes, and embedded in paraffin. Sections of 3 μm thickness were prepared and stained with H&E or aluminum staining.

2.2.4 Aluminum staining of dissected tumor sections

Paraffin sections were deparaffinized and stained with Einarson's reagent overnight. Stained sections were placed in 0.5% phloxine B for 3min and the excessive dye was removed by washing. Then, the sections were immersed in 5% phosphotungstic acid for 1min and subsequent 1% glacial acetic acid for 2min, followed by washing with 80% ethanol till the sections appear translucent. Sections were further replaced in 1% glacial acetic acid for 1min and counterstained with 0.05% fast green FCF for 3min. After dehydration of the sections with 95% and 100% ethanol (three changes each), coverslips were mounted.

2.2.5 Western blot analysis

Tumor samples were minced and lysed in a buffer containing 1% Nonidet-P40 supplemented with a complete protease inhibitor cocktail (Roche, Indianapolis, IN) and 2 mmol/L dithiothreitol. Lysates were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the following primary antibodies: rabbit polyclonal anti-ER α (sc-543; Santa Cruz, Dallas, TX), rabbit monoclonal anti-PUMA (12450; Cell Signaling Technology Inc., Danvers, MA), rabbit monoclonal anti-

Bak (12105; Cell Signaling Technology Inc.), rabbit polyclonal anti-Cox2 (ab15191; Abcam, Cambridge, UK), rabbit polyclonal anti-EP1 (101740; Cayman, Ann Arbor, MI), rabbit polyclonal anti-EP2 (101750; Cayman), rabbit polyclonal anti-EP3 (101760; Cayman), rabbit polyclonal anti-EP4 (101775; Cayman), mouse monoclonal anti- β -Actin (3700; Cell Signaling Technology Inc.), mouse monoclonal anti-GAPDH (MAB374; Millipore, Billerica, MA). After immunoblotting with secondary antibodies, proteins were detected with enhanced chemiluminescence (ECL) reagent (RPN2106, GE Healthcare, Buckinghamshire, UK).

2.2.6 Proliferation assay

The MCF-7 cells (1.5×10^6 /well) were seeded in 6-well plates and treated with 10 μ g/mL or 50 μ g/mL of STB-HO for 72 hours. To calculate the number of viable cells after STB-HO treatment, I performed trypan blue exclusion test, followed by the measurement of protein concentration from lysed cells and the bromodeoxyuridine incorporation assay to confirm the cell proliferation. BrdU proliferation kit was purchased from Roche.

2.2.7 Apoptosis assay

To evaluate the apoptotic rate, commercially available Apoptosis Detection Kit (BD biosciences, San Jose, CA) was used. After the indicated treatment of STB-HO, the cells were washed twice with PBS and resuspended in 100 μ L of binding buffer at a concentration of 1×10^5 and then 5 μ L of FITC-conjugated annexin V and 5 μ L propidium

iodide (PI) were added. The mixtures were gently vortexed and incubated for 15 minutes at room temperature. 400 μ L of binding buffer was added to the mixtures, and all samples were analyzed by flow cytometry within an hour, which was performed on a FACS Calibur using the Cell Quest software (BD biosciences)

2.2.8 Flow cytometric assay

For the characterization and analysis of markers on cell surface, flow cytometric analyses were performed using a FACS Calibur and the Cell Quest software (BD Biosciences). STB-HO population could be discriminated from MCF-7 cell population on a dot plot image based on the size of particles and cells. MCF-7, DU145, PC3, U87 and MDA-MB-231 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-ABC antibody (BD Biosciences). Nonspecific isotype-matched antibodies served as controls. For analysis of DC activation, DCs were incubated with Peridinin Chlorophyll (PerCP)-conjugated anti-CD83 antibody (BD Biosciences). NK cells were stained with FITC-anti-CD3 and PE-anti-CD56 antibodies to determine the purity of isolation. Antibodies for NK cell characterization were purchased from BioGems (Westlake Village, CA). All samples were incubated for 30 minutes at room temperature in the dark after Fc receptor blocking.

2.2.9 Cytokine production

Briefly, cells were treated with STB-HO for 48 hours and culture supernatant was harvested. Production of PGE₂, IL-10, IL-6 and IL-8 from MCF-7 cells in response to STB-

HO was measured in culture media using a commercial enzyme-linked immunosorbent assay kit (R&D Systems). Concentration of TNF- α , IL-1 β and IL-12 released from macrophages or DCs as well as NK cell production of IFN- γ were determined by a commercial kit (R&D Systems).

2.2.10 Differentiation of THP-1 into macrophage-like cells

To generate macrophage-like cells, THP-1 cells (2.5×10^5 /mL) were treated with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 hours. Differentiation of PMA treated cells was stabilized by culturing the cells for additional 5 days in fresh media without PMA. Differentiated cells were treated with STB-HO for 2 days and culture media was harvested and determined for cytokine production.

2.2.11 Isolation and culture of human umbilical cord blood (UCB)-derived mononuclear cell (MNC)

UCB samples were obtained from the umbilical vein immediately after delivery, and the informed consent of the mother was given and approved by the Boramae Hospital Institutional Review Board (IRB) and the Seoul National University IRB (IRB No. 1109/001-006). The UCB samples were mixed with Hetasep solution (StemCell Technologies, Vancouver, Canada) at a ratio of 5:1 and then incubated at room temperature to deplete erythrocyte counts. The supernatant was carefully collected and mononuclear cells were obtained using Ficoll density-gradient centrifugation at 2,500 rpm for 20 minutes. For maintenance of mononuclear cells, the cells were seeded in growth media that consisted

of RPMI1640 (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum. The cells were further used for the generation of macrophages, DCs and NK cells.

2.2.12 Generation of macrophage from human MNCs

CD14⁺ monocytes from UCB-derived MNCs were isolated using the immunomagnetic selection via CD14 Microbeads (Milteny Biotech, Auburn, CA) according to the manufacturer's protocol, yielding an average 98% purity. Briefly, 1×10^8 mononuclear cells were labeled with 200 μ L CD14 Microbeads and incubated in isolation buffer for 30 minutes at 4 °C. Cells were washed and resuspended with isolation buffer and the CD14⁺ cells were separated with an LS column (Milteny Biotech) placed on a column adapter in a strong magnetic field. The isolated cells were determined for their purity using flow and then gently resuspended in macrophage induction medium, consisting of RPMI1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 4 mmol/L GlutaMAX™ -I (Gibco BRL). Macrophages were obtained by culturing CD14⁺ monocytes for 7 days in 6-well cell culture plates (Nunc, Roskilde, Denmark) at a density of 1.5×10^5 /cm². Cells were refed with fresh culture medium every 3 days. At day 7, the adherent cells were treated with STB-HO for 48 hours and supernatant was harvested and analyzed for cytokine production.

2.2.13 Generation of DCs from human MNCs

To generate DCs, CD14⁺ monocytes were isolated from UCB-derived MNCs as previously described in macrophage generation. Isolated cells were seeded at a density of

2×10^6 cells/mL in 24-well plates with RPMI1640 (Gibco BRL) media containing 100 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, New Jersey, USA), 50 ng/mL human recombinant IL-4 (Peprotech) and 10% fetal bovine serum (Gibco BRL). After 3 days, fresh complete media was replaced. On day 5, non-adherent cells were removed and replaced with fresh media containing LPS (1 μ L/mL) and IFN- γ (20 ng/mL) or STB-HO to induce DC maturation. To evaluate DC maturation, CD83 expression on the surface of DCs was analyzed by flow cytometry.

2.2.14 Isolation of NK cells from human MNCs

NK cells were isolated from UCB-derived MNCs using the NK cell isolation kit according to manufacturer's instruction (#130-092-657, Miltenyi Biotec). Then, isolated NK cells were stained with FITC-anti-CD3 and PE-anti-CD56 antibodies for flow cytometric analysis and further used for NK cell cytotoxicity test.

2.2.15 NK cell cytotoxicity assay

MCF-7 cells were labeled with CFDA SE Cell Tracer Kit (V12883, Invitrogen, Eugene, OR) and co-culture with isolated NK cells in the presence of STB-HO or IL-12 cells among CFDA-labeled cells were detected to analyze the ratio of dead cells in MCF-7 cells. MCF-7 population could be discriminated from NK cell population based on the size, density and fluorescent intensity of cells.

2.2.16 Detection of NK cells in mouse spleen and lymph nodes

STB-HO (70 mg/kg) was orally administered into athymic nude mice and BALB/c mice for 2 wks. After sacrifice, spleen and lymph nodes were collected. Splenocytes were harvested from spleen by gentle mincing and filtration through the strainer. Cells were isolated from lymph nodes by mincing and subsequent digestion with type IV collagenase (0.5 mg/mL) for 30 minutes at 37 °C. Harvested cells were washed with PBS, followed by the incubation with PE-conjugated anti-NKp46 antibody (BioGems) for 30 minutes at room temperature in the dark. Stained cells were analyzed by flow cytometry.

2.2.17 Statistical analysis

All experiments were conducted at least three times ($n = 3$), and the results are expressed as the mean \pm SD. All statistical comparisons were made using one-way ANOVA followed by the Bonferroni post hoc test for multigroup comparisons using the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Statistical significance designated as asterisks is indicated in the figure legends.

2.3 RESULTS

2.3.1 Tumor growth reduction of MCF-7 xenograft model in the presence of STB-HO

To validate anti-cancer effects of Mica, I investigated the impact of STB-HO on breast cancer cell growth in mouse xenograft model. MCF-7 cells were subcutaneously transplanted into athymic nude mice. After 1 week, tumor volume was measured twice a week while two different concentrations of STB-HO were orally administered into mice for 12 weeks. Daily administration of STB-HO did not exert any adverse effects such as body weight loss, abnormal behavior and sudden death, indicating that the compound does not have toxicity in vivo. I observed that the tumor size did not as much grow in both low- and high-concentration of STB-HO-treated mice as normal saline-treated mice (Fig. 1A and B). After 12 weeks of oral administration, tumor volume in STB-HO-treated group was significantly lower compared to normal saline-treated group (Fig. 1C). Consistently, the volume and the mass of isolated tumors from sacrificed mice were significantly suppressed by STB-HO treatment in a dose dependent manner (Fig. 1D-F).

Upon histological examination of isolated tumor, massive infiltration of lymphocytes was observed at marginal location of tumor tissue from high concentration STB-HO-treated group, implying the potential of immunostimulatory effects by STB-HO (Fig. 2A). As estrogen receptor α (ER α), prostaglandin E2 (PGE2) produced by cyclooxygenase-2 (COX-2) and individual E-series of prostaglandin (EP) receptors are

known to play critical roles in breast cancer cell pathogenesis, progression, malignancy and metastasis (Fulton et al., 2006; Kang et al., 2014), I assessed the expression of each protein in tumor tissue. I observed the dose-dependent reduction in the expression of ER α in tumor from STB-HO-treated group. Furthermore, treatment of high dose STB-HO led to the decrease in COX-2 expression (Fig. 2B). STB-HO treatment also down-regulated the expression of four types of receptor for PGE₂, designated as EP 1–4 (Fig. 2C). I next explored the distribution of STB-HO in tumor tissue using the specific staining for aluminum, one of the STB-HO constituents. However, I couldn't detect any significant difference in the staining of tumor sections from STB-HO-administered group compared to normal saline-treated group (Fig. 3). Taken together, these results indicate that STB-HO administration can suppress the tumor growth and the expression of crucial factors for tumor progression and metastasis.

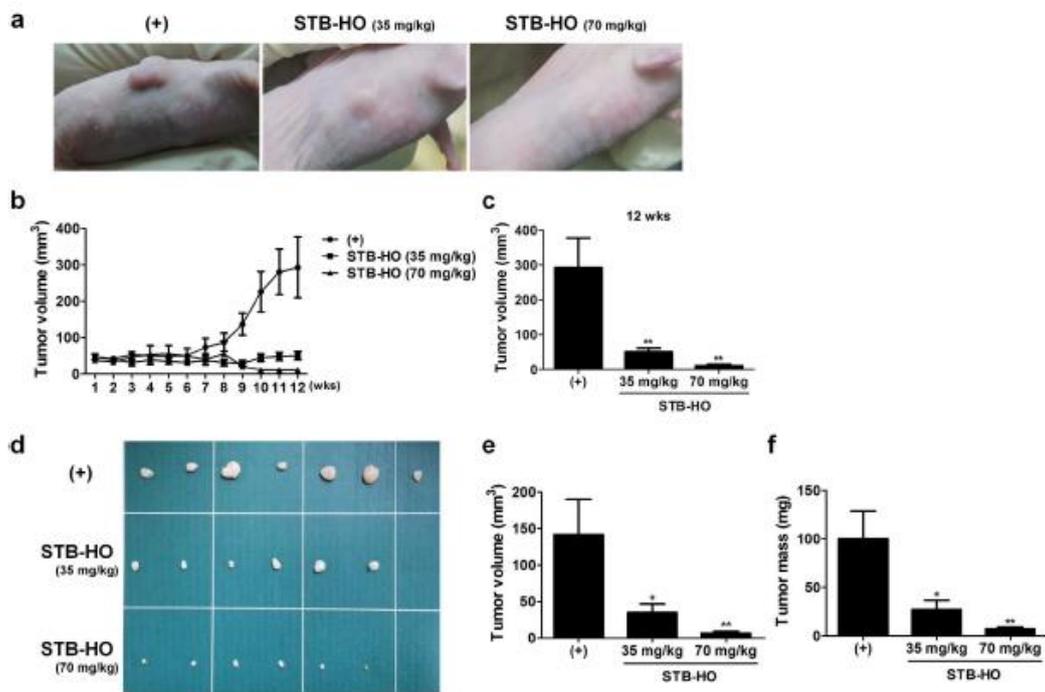


Figure 1. Tumor suppressive effect of STB-HO in MCF-7 xenograft mouse model.

(A-C) From 1 week of stabilization period after MCF-7 xenotransplantation, STB-HO was orally administrated daily in two different dose (35 mg/kg or 70 mg/kg) for 12 weeks and tumor size was measured twice a week. (A) Images of tumors in mice. (B) Tumor volume measurement in a time course. (C) Tumor volume at 12 weeks. (D-F) Tumors from xenograft mouse model were isolated and measured. (D) Images of isolated tumors. (E) Volume and (F) Mass of isolated tumors. Seven to eight mice per group were used. *P < .05, **P < .01. Results are shown as mean \pm SD.

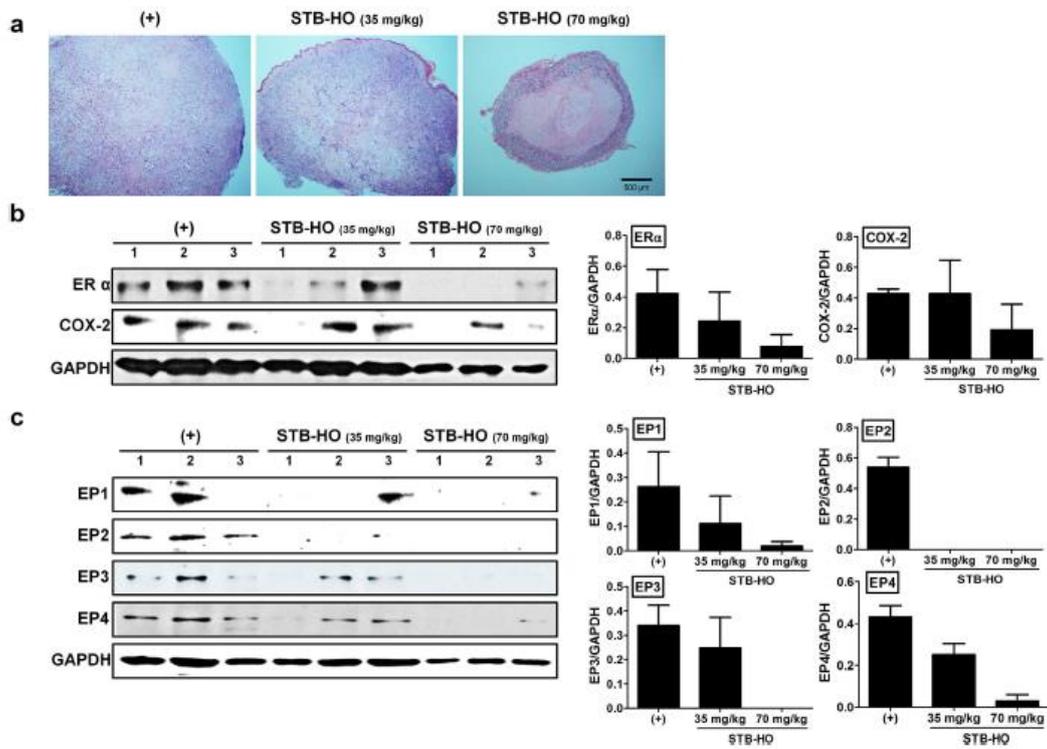


Figure 2. Signal regulation effect of STB-HO in MCF-7 tumor formation.

Isolated tumors were processed for further histological analysis and specific protein detection. (A) Dissected tumors were stained with hematoxylin and eosin. (B-C) Tumors were lysed and analyzed for the expression of crucial proteins in tumor growth. (B) ER α and COX-2 expression in tumor samples were detected by western blotting and quantified. (C) Expressions of four EP receptors were determined and quantified. Results are shown as mean \pm SD.

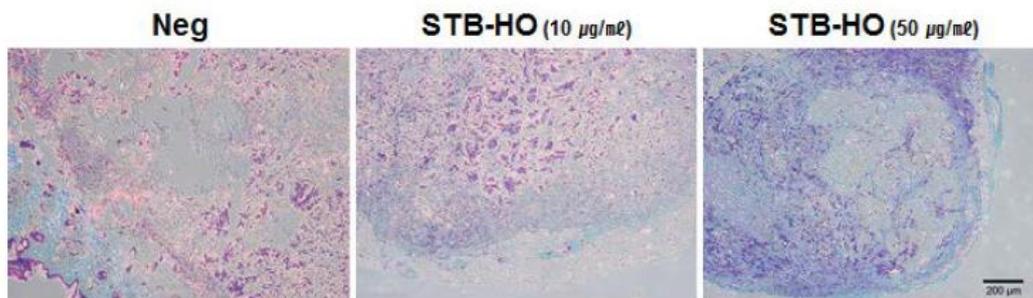


Figure 3. Tracking of STB-HO in tumor tissue.

Aluminum staining was performed to determine the distribution of orally administered STB-HO in tumor tissue.

2.3.2 STB-HO does not directly regulate the proliferation and viability of MCF-7 cells in vitro

To verify the mechanism of STB-HO action on breast cancer cells, I investigated whether treatment of STB-HO can affect the proliferation or apoptosis of various cancer cells including MCF-7 cells. Phagocytosis of STB-HO particles by cancer cells was detected by time-lapse microvideography. After 72 hours of treatment, STB-HO inhibited the proliferation of cancer cells including prostate cancer (DU145 and PC3), glioblastoma (U87) and breast cancer (MDA-MB-231), whereas unexpected slight inhibition was observed in the proliferation of MCF-7 cells when same concentration of STB-HO was treated (Figure 4A-D and Fig. 5). Similarly, STB-HO treatment didn't induce the apoptosis of MCF-7 cells nor increase the expression of apoptosis-related signals while those signals were activated in prostate cancers and glioblastoma in response to STB-HO (Fig. 4E, F and Fig. 5). On the otherhand, to evaluate the *in vitro* toxicity of STB-HO, I treated STB-HO on human dermal fibroblasts and observed that the proliferation or the apoptosis of fibroblasts were not affected by the treatment (Fig. 5). These findings suggest that STB-HO does not have direct effect on the growth of MCF-7 cells, and that indirect mechanisms might be involved in the anti-tumor effect of STB-HO in xenograft model.

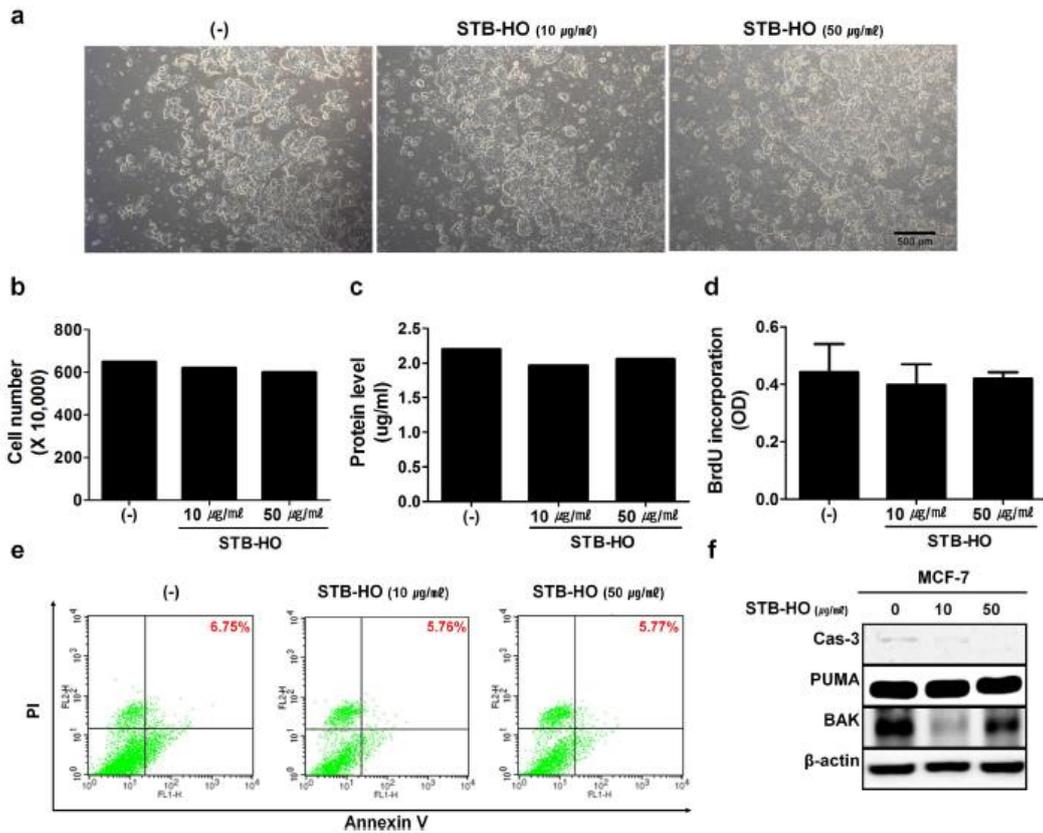


Figure 4. Direct effects of STB-HO treatment on MCF-7 cell proliferation and apoptosis.

(A) MCF-7 cells were treated with two different concentrations of STB-HO for 3 days and images were taken. (B-D) MCF-7 proliferation in response to STH-HO was determined with various methods (B) A trypan blue exclusion test was performed. (C) Concentration of total protein from lysed cell was measured. (D) BrdU incorporation assay was performed. (E) Apoptotic rate in MCF-7 cells was measured by staining Annexin V after STB-HO treatment. (F) Apoptosis-related proteins were detected by western blot analysis. Results

are one representative experiment of three independent experiments. Results are shown as mean \pm SD. Cas-3; caspase-3, PUMA; p53 upregulated modulator of apoptosis, BAK; Bcl-2 homologous antagonist/killer.

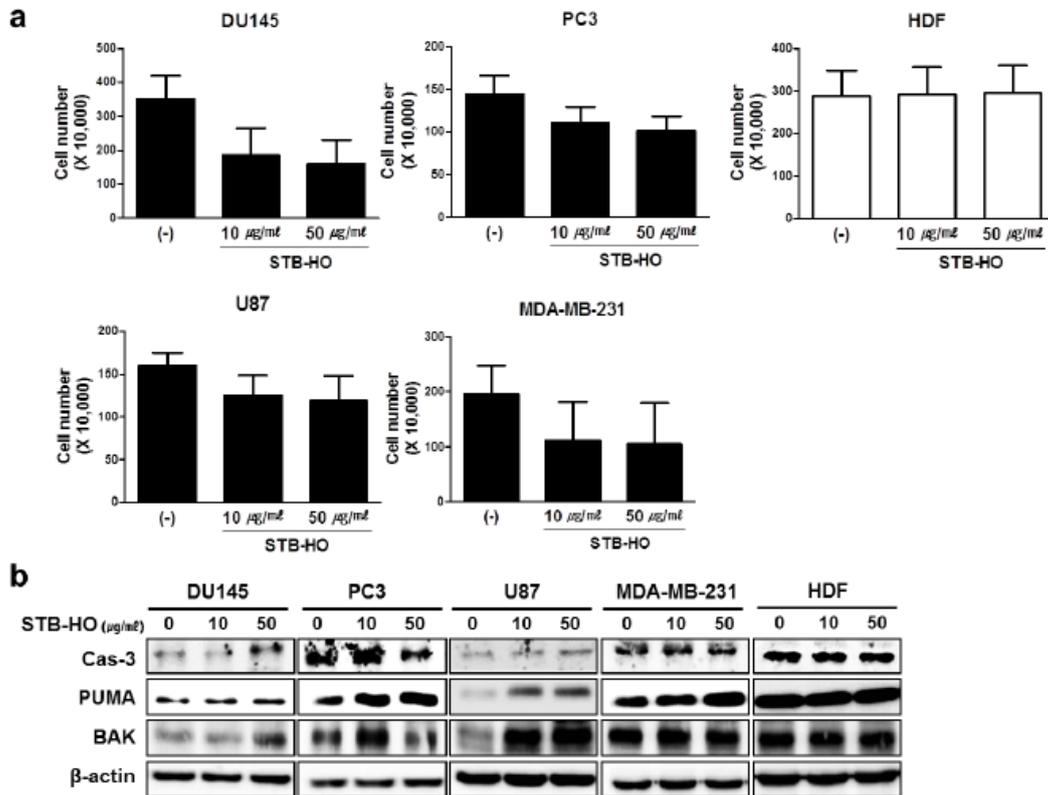


Figure 5. Effects of STB-HO treatment on various cancer cells and fibroblasts.

(A) Prostate cancer cells (DU145 and PC3), glioblastoma (U87), breast cancer cells (MDA-MB-231) and human dermal fibroblasts (hDFs) were treated with STB-HO. After 72 hours, a trypan blue exclusion test was performed to verify the proliferation of cells. (B) Proteins from cancer cells and hDFs were analyzed by western blot to identify the expression of apoptosis-related markers.

2.3.3 STB-HO increases the susceptibility of MCF-7 cells to their Microenvironment

Given that the anti-tumor effect of STB-HO might involve in indirect mechanisms rather than direct inhibitory effect on cancer cell growth, I next examined whether STB-HO can regulate the evasive behavior of MCF-7 to avoid the attack by immune system. HLA class I molecule is a well-known inhibitory factor for NK cell-mediated anti-tumor effect. Accordingly, it has been reported that NK cells can kill target cells expressing low levels of HLA class I molecule (Majid et al., 2005). Therefore, I first examined the alteration in the expression of HLA class I molecule by STB-HO treatment. Interestingly, while 24% of MCF-7 cells expressed MHC-expressed class I antigens, HLA-ABC, on cell surface, STB-HO treatment down-regulated the expression of these antigens to approximately 10% (Fig. 6A). This inhibitory effect of STB-HO on the expression of MHC class I was consistently observed in other types of cancer cells (Fig. 7). In addition, because tumor cells are reported to use immunomodulatory soluble factors such as IL-6, IL-8, IL-10 and PGE2 for immune evasion (Chikamatsu et al., 2011; Fisher et al., 2014; Orleans-Lindsay et al., 2001; Shields et al., 2010; Wang and Dubois, 2010), I next detected the concentration of these soluble factors secreted by MCF-7 cells after STB-HO treatment. The concentration of PGE2 in MCF-7 culture media was significantly reduced by STB-HO treatment in a dose-dependent manner, whereas other cytokines were hardly detectable (Fig. 6B). Taken together, these results indicate that STB-HO treatment attenuates the immune evasive ability of MCF-7 cells by impairing their production of inhibitory factors.

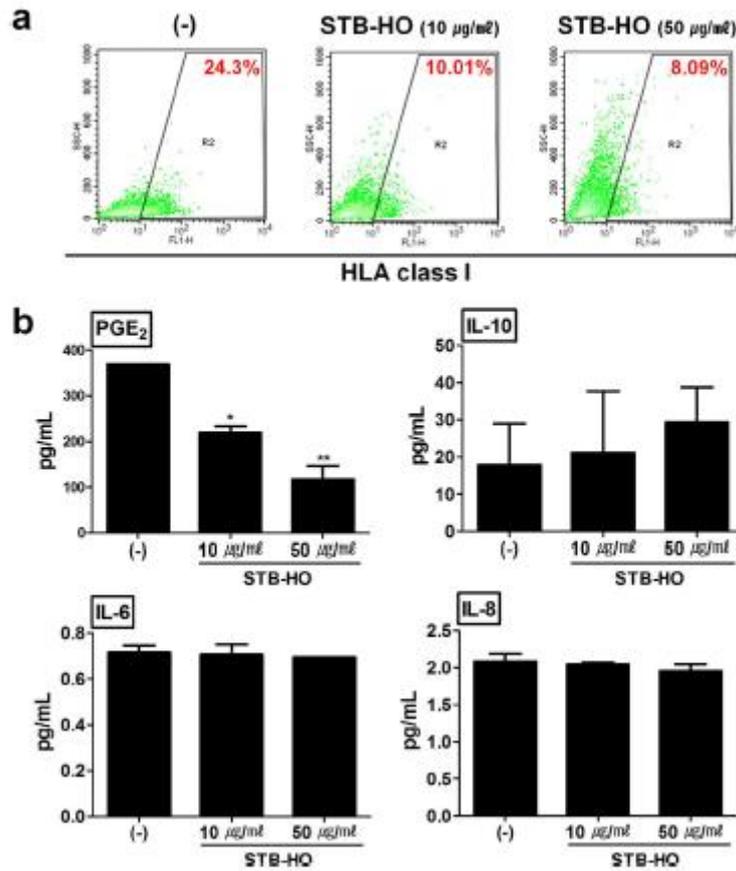


Figure 6. Regulation of immune evasive components in MCF-7 cells by STB-HO treatment.

(A) The expression of HLA class I on the surface of MCF-7 cells was detected by flow cytometry after STB-HO treatment. (B) The secretion of soluble factors related with immune evasion was measured using commercial kits. Production of PGE₂, IL-10, IL-6 and IL-8 from MCF-7 cells was detected from culture supernatant. *P < .05, **P < .01. Results are one representative experiment of three independent experiments. Results are shown as mean \pm SD.

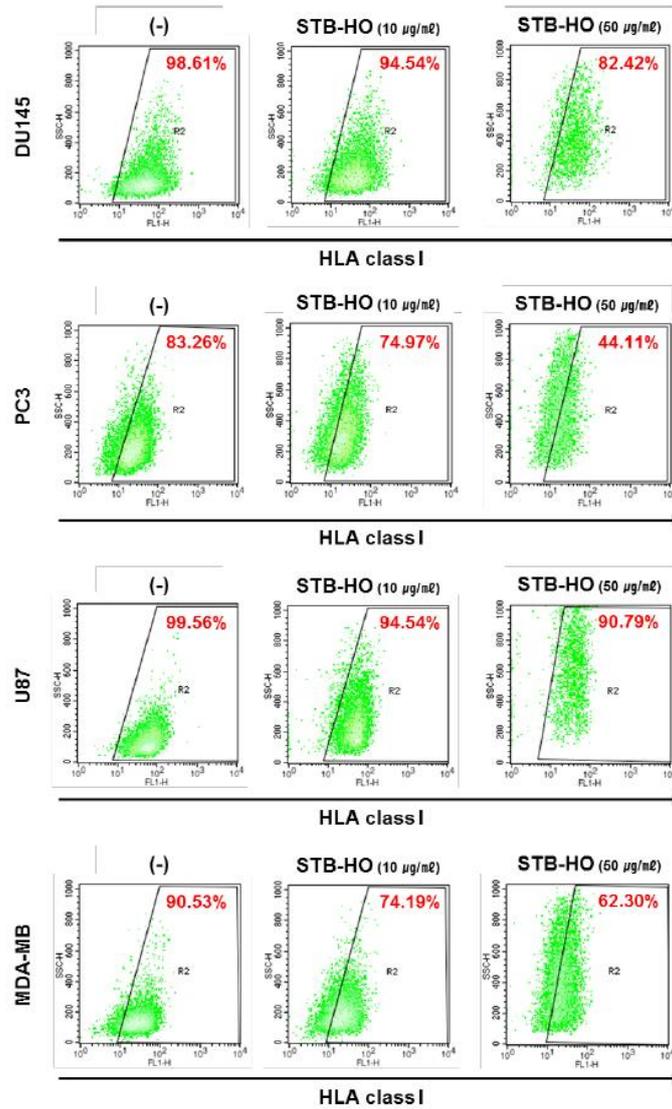


Figure 7. Effects of STB-HO treatment on the expression of HLA class I in various cancer cells.

The expression of HLA class I on the surface of DU145, PC3, U87 and MDA-MB-231 was detected by flow cytometry after STB-HO treatment.

2.3.4 STB-HO skews macrophages and dendritic cells toward anti-tumor type.

Since I found that STB-HO increased the susceptibility of MCF-7 cells to immune cells, I then investigated the effects of STB-HO on major cells of anti-tumor immunity. I first analyzed the functional alteration in macrophages and dendritic cells in response to STB-HO. Macrophage-like cells were induced from THP-1, followed by STB-HO treatment (Fig. 8A). I observed that macrophages engulfed STB-HO (Fig. 8B) and their secretion of TNF- α , IL-1 β and IL-12, prominent cytokines of M1 type macrophage, were augmented by STB-HO treatment (Fig. 8C). Among these cytokines, IL-12 is known to critically contribute to the NK cell cytotoxicity against tumor cells. Therefore, I confirmed the consistent increase in IL-12 production by STB-HO treatment using primary cultured macrophages (Fig. 8D). DCs were prepared from CD14⁺ monocytes and determined for STB-HO-induced activation (Fig. 9A). To analyze the activation of DCs, I stained cells with CD83, one of the surface markers indicating DC activation. I observed that high concentration of STB-HO increased CD83 expression up to 44% compare to 26% in naive DCs (Fig. 9B). Consistently, IL-12 secretion of DCs in response to STB-HO was enhanced (Fig. 9C). However, low concentration of STB-HO did not alter the expression of DC activation marker, implying that appropriate dose of STB-HO for the activation of macrophages or DCs might be distinct.

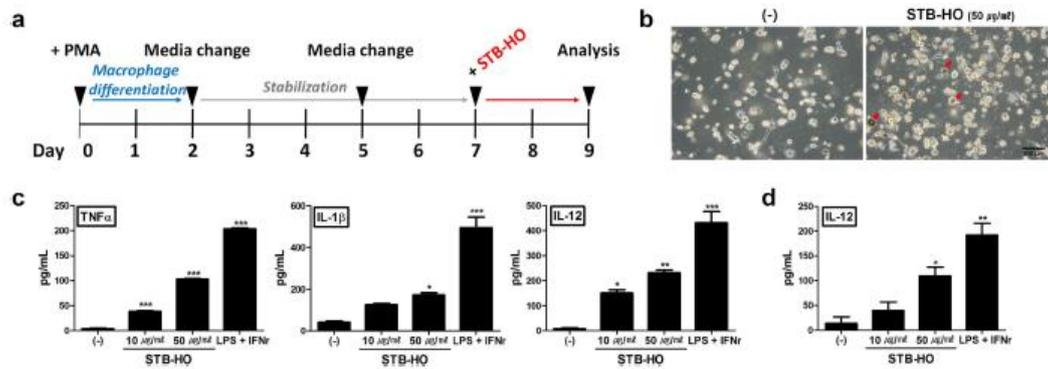


Figure 8. Polarization of macrophages by STB-HO treatment.

(A) A time line for the differentiation of THP-1 into macrophage-like cells. (B) Photographs of THP-1-derived macrophages after STB-HO treatment. STB-HO engulfed by macrophages is indicated by red arrow. (C) TNF- α , IL-1 β and IL-12 secretion was measured in culture media of THP-1-derived macrophages. (D) Production of IL-12 from MNC-derived macrophages was detected. Treatment of LPS with IFN- γ was used to induce the polarization of macrophages. *P < .05, **P < .01, ***P < .001. Results are one representative experiment of three independent experiments. Results are shown as mean \pm SD.

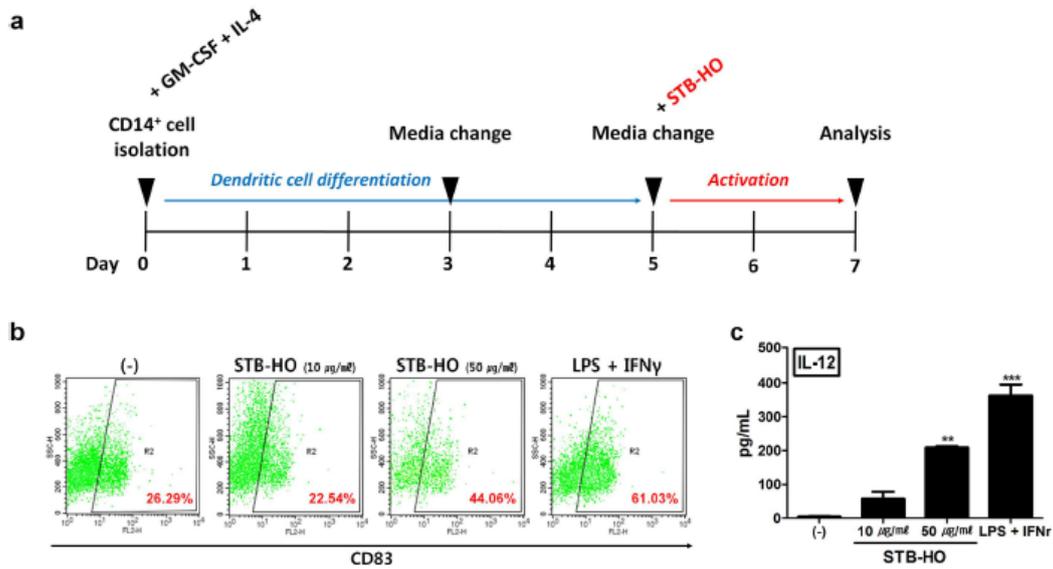


Figure 9. Activation of DCs by STB-HO treatment.

(A) Outline for DC induction from human MNCs. (B) The expression of CD83, DC activation marker, was detected by flow cytometry after STB-HO treatment. (C) IL-12 concentration in culture media of DCs was measured. Treatment of LPS with IFN- γ was used to induce the maturation of DCs. **P < .01, ***P < .001. Results are one representative experiment of three independent experiments. Results are shown as mean \pm SD.

2.3.5 STB-HO up-regulates the NK cell-mediated killing of MCF-7 cells in vitro and increases the number of NK cells in vivo

To explore the effect of STB-HO on NK cells, a major component of the anti-tumor immunity, I isolated NK cells from human blood and evaluated the purity of isolated cells by targeting CD3- CD56+ cell population (Fig. 11A). After STB-HO treatment, the secretion level of IFN- γ from isolated NK cells was significantly elevated to the extent that was observed in cells activated by IL-12 as a positive control group (Fig. 11B). To better demonstrate the actual cytotoxicity of activated NK cells against MCF-7 cells, I performed cytotoxicity test by co-culturing NK cells with fluorescence-labeled MCF-7 cells (Fig. 10). I observed that 2% of dead population in MCF-7 cells was increased up to 7% in the co-culture condition. Interestingly, the proportion of dead cell was significantly raised up to 16–17% when STB-HO was added to co-culture condition (Fig. 11C). Furthermore, the concentration of IFN- γ in co-culture media showed significant elevation following the STB-HO treatment (Fig. 11D).

Based on these *in vitro* results showing the NK cell-activating ability of STB-HO, I further investigated whether similar effect can be observed by oral STB-HO administration *in vivo*. To this aim, athymic nude mice and BALB/c mice were orally administered with 70 mg/kg STB-HO daily for 2 weeks, and the population of NKp46+ cells was determined. I observed significant increase in NKp46+ cells in spleens of both athymic and BALB/c mice while cell population in lymph node was not changed (Fig. 12).

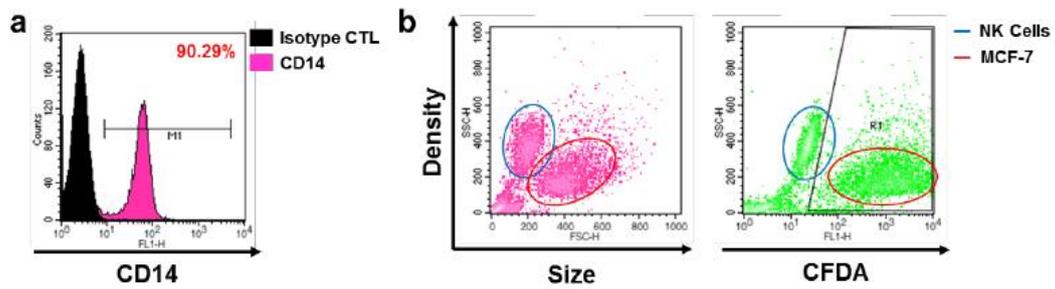


Figure 10. Characterization of sorted cells and gating strategy for flow cytometry to discriminate MCF-7 cells from NK cells.

(A) To generate macrophages and dendritic cells, CD14⁺ monocytes were isolated from mononuclear cells and their purity was determined by flow cytometry. (B) Size, density and fluorescence intensity of NK cells with CFDA-labeled MCF-7 cells were detected by flow cytometry. The population in blue and red ellipse indicates NK cells and MCF-7 cells, respectively.

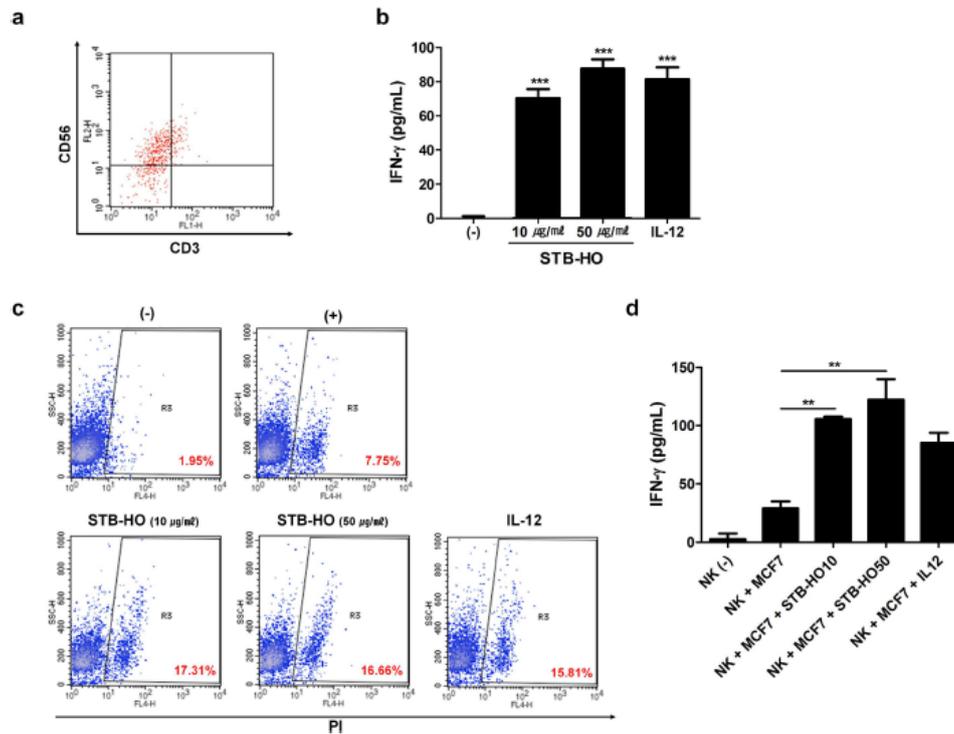


Figure 11. Enhancement of NK cell cytotoxicity upon STB-HO treatment.

(A) Isolated NK cells were determined for the purity through the detection of CD56 and CD3 on cell surface using flow cytometry. CD3- CD56+ cells represent NK cells. (B) IFN- γ secretion from isolated NK cells was measured. (C, D) CFDA-labeled MCF-7 cells were co-cultured with NK cells in the presence of STB-HO for 4 hours. (C) Co-cultured cells were stained with PI and PI-expressing dead cells were detected by flow cytometry. (D) Culture media used in co-culture was determined for the production of IFN- γ . IL-12 treatment was used to induce the activation of NK cells. **P < .01, ***P < .001. Results are one representative experiment of three independent experiments. Results are shown as mean \pm SD.

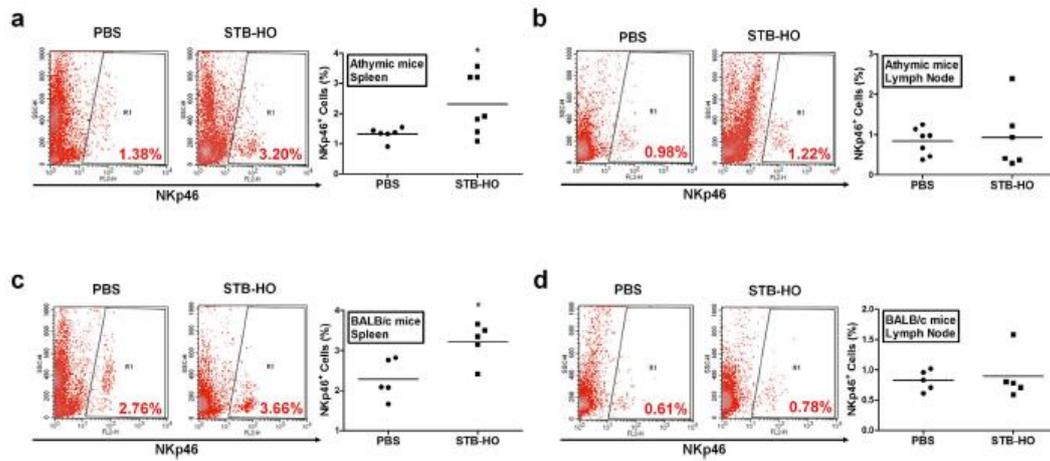


Figure 12. Increase in the number of NK cells residing in mouse spleen by STB-HO administration.

(A-D) The alteration in the number of NK cells in mice after oral STB-HO administration was determined. The cells from spleen and lymph nodes of (A, B) athymic nude mice or (C, D) BALB/c mice were isolated and NKp46+ cells in isolated cells were detected by flow cytometry. Five to seven mice per group were used. *P < 0.05.

2.4 DISCUSSION

In the present study, I investigated the anti-tumor effect of STB-HO against MCF-7 breast cancer cells. STB-HO administration efficiently suppressed the tumor growth of MCF-7 in xenograft model. These results are consistent with the previous study by Cho *et al.*, suggesting the suppressive capability of STB-HO on the growth of HCT colorectal cancer cells (Cho *et al.*, 2013). Similarly, a number of studies have provided evidences that minerals have anti-tumor potential in breast cancer cells (Brama *et al.*, 2007; Xia *et al.*, 2012). The majority of these studies showed that treatment of minerals including STB-HO on cancer cells lead to the inhibition of cell growth or the induction of apoptosis through the regulation of crucial receptors or signaling. However, in this study, STB-HO treatment didn't exert direct inhibitory effects on MCF-7 proliferation nor induce the apoptosis while STB-HO suppressed the proliferation of other types of cancer cells. Interestingly, the proliferation of MDA-MB-231, another type of breast cancer cells, was inhibited by the treatment of STB-HO. This discrepancy between two breast cancer cells might result from differences in their nature such as the expression of ER, crucial signaling pathway and the capability to repair DNA. In general, MDA-MB-231 cells are known to be more sensitive to anti-tumor drug treatment, which is consistent with our findings. However, to precisely explain the underlying mechanisms, the composition of STB-HO is not yet fully discovered. Therefore, a future study to explore the constituents of minerals should be preceded to address this discrepancy in the inhibitory mechanisms. These findings were interesting

since STB-HO treatment *in vivo* led to the significant inhibition of tumor growth and down-regulation of growth & metastasis-related signaling in MCF-7 graft model. Instead, STB-HO treatment decreased the expression of HLA class I molecule, a well-known inhibitory ligand for NK cell function, on the surface of MCF-7 cells, indicating that cancer cells exposed to STB-HO might be more susceptible to NK cell cytotoxicity. This suggestion is based on the previously reported studies proposing that various types of tumor cells become susceptible to NK cell-mediated killing when the expression of HLA class I molecules is defective or lower (Brandt et al., 2009; Castriconi et al., 2009; Pende et al., 2005). Moreover, STB-HO impaired the secretion of PGE2 from MCF-7 cells and inhibited the expression of PGE2-producing enzyme, COX-2. Because PGE2 is known to contribute to tumor growth, apoptotic resistance, angiogenesis and invasiveness (Fosslien, 2000; Greenhough et al., 2009), one can envision that suppressed production of PGE2 by STB-HO treatment can make MCF-7 cells more susceptible to their microenvironment including NK cells.

Mica particles have been continuously developed by several researchers to establish the most effective formulation as feed supplements, and their functions have been explored for application (Aikoh et al., 1998; Holian et al., 1997; Jung et al., 2013; Jung et al., 2010; Jung et al., 2015a). The most recent study by Jung *et al.* reported that the newest form of mica, STB-HO, can activate the macrophage which contributes to the immunoenhancement (Jung et al., 2015b). Consistently, I here demonstrated that STB-HO activates the macrophage by skewing them toward immunostimulatory M1 phenotype, which secretes TNF- α , IL-1 β and IL-12. More importantly, since IL-12 signaling is

reported to be critical for the effector function of NK cells (Simhadri et al., 2014), the present study found the evidence to associate previous results regarding mica-mediated macrophage activation with anti-tumor effects. Similarly, DCs as well as macrophages produced higher level of IL-12 in response to STB-HO treatment, along with the augmented expression of surface marker for DC activation. Although NK cell is the major component to attack tumor cells, none of previous studies using STB-HO or its prototype explored the influence of mica on NK cell function. I revealed that STB-HO treatment on NK cells generates IFN- γ -secreting effector cells that are capable of killing MCF-7 cells in co-culture system. IFN- γ is a well-known cytokine that polarizes macrophages toward M1 type (Gordon and Martinez, 2010; Mantovani et al., 2002). Our findings, taken together with previous reports, suggest that NK cells activated by M1 macrophage, in turn, can shape functional behavior of macrophages toward NK stimulatory capability. Since athymic nude mice possess NK cells and macrophages but not T lymphocytes, it seems plausible that my findings of STB-HO-stimulated NK cell cytotoxicity might be a key mechanism for *in vivo* anti-tumor effect. Finally, this study also confirmed that STB-HO administration *in vivo* can actually increase the number of NK cells in spleen of both athymic mice and BALB/c mice. These data support the possibility that STB-HO could be applied for NK cell-mediated immunotherapy against tumor by increasing the absolute number of NK cells, as the insufficient number of NK cells is one of the major impediments for efficient treatment (Vujanovic et al., 1996). Although STB-HO has been developed as a feed supplement, securing safety and distribution of the compound is indispensable for clinical use. In this study, no adverse effects were observed in mice during whole period of

daily STB-HO administration. Additionally, STB-HO didn't exert any effects on proliferation and apoptosis of human fibroblast, a representative normal tissue cell. These findings are consistent with the previous study by Cho *et al.*, showing that mica particles did not show any cytotoxicity in human umbilical vein endothelial cells (Cho et al., 2013). I further proved that STB-HO didn't have any harmful effects on human adult stem cells (data not shown). However, I couldn't uncover the distribution pattern of STB-HO following oral administration into mice, even though I performed several staining techniques using fluorescence and aluminum staining. Therefore, it is apparent that future work will be required to investigate the distribution and excretion of STB-HO by developing or applying more sensitive techniques of particle detection.

Taken together, the present study revealed novel information implying that mica nanoparticles increase the susceptibility of breast cancer cells to their microenvironment and help microenvironment-constituting NK cells and supporting DCs or macrophages to attack cancer cells. My findings are expected to provide a basis for development of an alternative therapeutics to treat tumor cells using natural compounds.

GENERAL CONCLUSION

This study showed two different treatment strategies using a novel small molecule as a targeted therapeutic agent and a natural compound as an immunotherapeutic agent. I investigated the dual anti-tumor effect of PDE4D inhibitor, CG500354, in human primary glioblastoma cells. I found that CG500354 specifically blocks PDE4D and upregulates cyclin-dependent kinases inhibitors p21 and p27 as well as their upstream target p53 to play growth arrest role in human primary GBM-derived cells. Moreover, CG500354 also triggered glioblastoma cells to differentiate into Tuj1- and GFAP-expressing neural cell types while nestin expression underwent down-regulation. I investigated that these effects of CG500354 accompany the increased level of cAMP followed by up-regulation of the phosphorylation of PKA and CREB. I indicated that CG500354 can alter the phenotypes of human primary GBM cells from an oncogenic state into a less oncogenic and more differentiated state. More interestingly, only CG500354 reduced nestin expressing population through treatment while well-known mimetic substances, Forskolin and Rolipram, could not. My findings suggest that CG500354 plays a pivotal role in human primary GBM and its functions may be even more effective than Forskolin and Rolipram in its dual anti-cancer potential.

In the second study, I investigated on a natural compound, STB-HO in several cancer cells, especially the breast cancer cells. HLA class I expressions were down-regulated due to STB-HO treatment, which indicates that STB-HO may cause cancer to be more susceptible to NK cell cytotoxicity. Moreover, STB-HO down-regulates the

expression of COX-2 by interrupting PGE2 secretion from breast cancer cells. Since PGE2 is known for its invasiveness, tumor growth and apoptotic resistance potential, my finding may indicate that STB-HO enhances susceptibility of MCF-7 cells to their microenvironment including NK cells. I here demonstrated whether STB-HO stimulates the macrophages to secrete TNF- α , IL-1 β and IL-12 by inducing them towards immunostimulatory M1 phenotype. My previous results suggest that STB-HO reveals its anti-tumor effect by activating macrophages. As predicted, DCs also secreted higher level of IL-12 in the presence of STB-HO, along with the augmented expression of surface marker for DC activation. Despite the fact that NK cell is the major component in assaulting tumor cells, none of the previous studies researched the enhancing role of STB-HO in NK cell function using STB-HO or its prototype. Thus, I performed co-culture system and revealed STB-HO treated NK cells produce IFN- γ secreting effector cells which have an ability to eliminate breast cancer cells. In addition, I confirmed that the number of NK cells in the spleen of both athymic mice and BALB/c mice were increased when STB-HO was orally administrated *in vivo*.

In summary, my research discovered that the novel small molecule, CG500354, and natural compound, STB-HO, have a potential to be developed into an alternative therapeutics for tumor cells by controlling the signaling pathways, and improving the susceptibility of cancer cells to their microenvironment by increasing NK cell constitution to attack the cancer cells.

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국문 초록

암세포에서 MICA 나노입자와 PDE4D 억제제의 종양 억제 연구

서울대학교 대학원

수의학과

협동과정 인수공통동물질병학 전공

강 태 옥

(지도교수: 강경선)

다형성교아종과 유방암은 전세계적으로 성인에서 많이 발생하는 암으로서 암세포의 증식력과 현재의 치료기술적인 한계로 인하여 높은 사망률을 보이고 있다. 본 연구에서는 새로운 소분자 물질인 CG500345과 천연 미네랄 나노입자인 STB-HO를 이용한 직접적인 종양억제효과와 면역증진을 통한 간

접적인 종양억제효과를 검증하고 각각에 대한 기전을 규명하기 위한 연구를 수행하였다.

첫 번째로, 최근 항암 치료방법으로 연구되고 있는 암세포의 분화에 대한 물질의 효과를 검증하기 위한 연구를 수행하였다. 해당 연구에서는 인산디에스테라아제-4 아형 D (PDE4D) 억제 물질인 CG500354가 cAMP/PKA 신호 경로의 활성을 일으켜 다형성교아종 (Glioblastoma Multiforme)의 성장 억제와 신경 분화를 유도한다는 것을 밝혔다. 즉, CG500354의 처치가 인산디에스테라아제-4 아형 D의 간섭을 방해함으로써 cAMP/PKA 신호 경로를 조절하여 인산화단백질키나아제 A (p-PKA)와 인산화 cAMP 반응순서결합단백질 (p-CREB)의 발현을 증가시켰다. 나아가, CG500354는 성장억제 관련 유전자인 p21과 p27의 mRNA 발현을 증가시키는 동시에 싸이클린 B1을 감소시킴으로서 교아종의 성장을 현저히 감소시켰다. 또한 면역세포화학 염색과 단백질 분석을 통해 CG500354 처치에 의해 정상세포 마커인 GFAP와 신경계 마커인 Tuj-1 그리고 암 억제 유전자인 p53 발현이 증가함과 동시에 신경간세포 마커인 Nestin의 발현이 현저히 감소하였음을 확인하였다. 이 결과는 CG500354가 세포주기 및 신경분화 조절기전을 통해 다형성교아종의 성장을 억제할 수 있음을 의미한다.

두 번째로, 알루미늄노규산염 미네랄인 운모, STB-HO가 종양억제 면역세포와 종양세포간의 상호작용에 미치는 영향을 규명하기 위한 연구를 진행하였다. MCF-7 세포가 이식된 쥐에 STB-HO를 경구투여 하였을 때, MCF-7

유방암세포주의 증식이 현저하게 억제됨을 확인하였다. 반면, 세포실험에서 해당 물질을 직접적으로 세포에 처치하였을 때는 증식억제 및 세포사멸 효과가 관찰되지 않았다. 따라서, 이와 같은 동물실험과 세포실험간의 서로 다른 결과 차이를 분석하기 위하여 종양 미세환경을 구성하는 세포로 알려진 대식세포, 수지상세포 그리고 자연살해 세포와 MCF-7세포간의 상호작용에 있어서 STB-HO 처치가 어떤 효과를 나타내는지를 연구하였다. STB-HO의 처치는 암세포가 자연살해세포의 공격을 회피하기 위해 발현하는 MHC class I의 발현을 10% 정도 억제하였다. 또한, STB-HO의 처치는 암세포를 더욱 효율적으로 공격할 수 있도록 대식세포와 수지상세포를 활성화시킴을 확인하였다. 또한, 혈액에서 자연살해세포를 직접 분리하여 MCF-7 세포와 공동배양함으로써 자연살해세포의 유방암 세포주에 대한 세포독성을 확인 하였다. 그 결과, 공배양 시, 7% 정도의 MCF-7 세포의 사멸 비율이 STB-HO의 처치에 의해 16-17% 수준까지 증가하며 동시에 배양액 내에 IFN- γ 의 농도가 현저하게 증가함을 확인하였다. 이 결과는 STB-HO가 MCF-7의 면역세포에 대한 저항성을 감소시킬 뿐만 아니라 종양 미세환경을 구성하는 면역세포의 항암효과를 증진시킴으로써 암세포를 사멸시킬 수 있음을 의미한다.

종합적으로, 본 연구를 통하여, 암세포의 성장억제를 위한 신규물질의 항암작용 기전을 규명하였으며 구체적으로 1) PDE4D 억제제인 CG500354는 cAMP/PKA 신호경로의 활성화를 통해 다형성교아종의 신경분화를 촉진하고 동시에 성장을 억제하며 2) STB-HO는 유방암세포의 면역저항 또는 회피

기능을 감소시키는 동시에 대식세포, 수지상세포 및 자연살해세포 등의 면역세포의 항암효과를 증진 시킴으로써 항암치료의 후보 물질로서 이용될 수 있음을 제시하였다.

주요어 : 다형성교아종, 인산디에스테라아제-4 아형 D 억제제, cAMP, 유방암, 운모, 종양억제 효과

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