International Journal of Biochemistry and Cell Biology 92 (2017) 155–163

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Research paper

The synergistic effects of valproic acid and fluvastatin on apoptosis induction in glioblastoma multiforme cell lines

Yung-Lung Chang[a], Li-Chun Huang[a], Ying-Chuan Chen[a], Yi-Wen Wang[b], Dueng-Yuan Hueng[a,c,*], Shih-Ming Huang[a,*]

[a] Department of Biochemistry, National Defense Medical Center, Taipei City 114, Taiwan, Republic of China
[b] Department of Biology and Anatomy, National Defense Medical Center, Taipei City 114, Taiwan, Republic of China
[c] Department of Neurological Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei City 114, Taiwan, Republic of China

ARTICLE INFO

Keywords:
Glioblastoma multiforme
Histone deacetylase inhibitor
Statin
Valproic acid
p21

ABSTRACT

Glioblastoma multiforme (GBM) is the most common primary central nervous system malignant tumor. It responds poorly to standard therapies, such as surgical resection, radiation therapy and chemotherapy. Many chemotherapeutic drugs are focused on apoptosis induction and radiation sensitivity. Inhibition of histone acetylation via histone deacetylase inhibitor (HDACI) is one such strategy. Statins (or 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) are classical drugs used to lower cholesterol but also inhibitors of histone deacetylation activity. This study analyzes the combinatory effects of valproic acid (VPA) and fluvastatin on apoptosis induction in GBM8401 cells. The results show that they act synergistically in inducing γ-H2AX and apoptosis accompanied by higher acetylated histones H3 and H4. Downregulation of p53 occurred by VPA alone and fluvastatin alone, but not at their combined application; upregulation of p21 at the protein level was induced by each of the drugs alone and no further increase occurred at combined application. The drug BEZ235 inhibited phosphorylation of Akt and attenuated the level of γ-H2AX as well as cleaved PARP (cPARP) induced at combined application of VPA and fluvastatin. Induction of apoptosis within a 48 h incubation period was massive when measured as the subG1 peak (97%) and was detected after a 24 h incubation at low level when assayed with PE Annexin V. Synergistic apoptosis induction was demonstrated also after 24 h incubation by the appearance of cPARP. Partial silencing of p21 reduced cPARP as well as the percentage of apoptotic cells in the subG1 peak. However, partial silencing of p53 had no effect on apoptosis. Such findings offer a better understanding of the mechanism of action of HDACIs in combination with statins that may guide the development of a new combinatory reposition for the treatment of GBM.

1. Introduction

Glioblastoma multiforme (GBM) is the most common life-threatening primary central nervous system tumor. Current treatment involves surgical resection, followed by radiation and chemotherapy. However, the median survival rate is only 8–15 months (Stupp et al., 2005). The standard chemotherapeutic agent is temozolomide (TMZ), an orally administered alkylating agent that is well absorbed and readily crosses the blood-brain barrier (BBB). The reported resistance to TMZ treatment in GBM is mediated via the reactivation of O^6-methylguanine-DNA methyltransferase (MGMT), leading to DNA replication and tumor(s) growth (Friedman et al., 2000; Gerson, 2004). Many approaches to overcome TMZ resistance have focused on the regulation of MGMT expression. Actually, GBM is characterized by rapid proliferation, high invasiveness and resistance to apoptosis (Louis, 2006). Hence, alternative treatment approaches should aim to inhibit cellular proliferation and induce apoptosis, using a drug repositioning strategy.

Histone deacetylases (HDACs) are among the most promising targets in drug development for cancer therapy. Acetylation and deacetylation of core histones are key regulatory mechanisms of gene expression (Khochbin et al., 2001). Aberrant histone deacetylation at tumor-suppressor genes occurs in various human tumors (Mahlknecht and Hoelzer, 2000; Cress and Seto, 2000). Various HDAC inhibitors (HDACIs) have been identified as promising compounds for cancer treatment, either alone (Eyupoglu et al., 2005; Wetzel et al., 2005) or in combination with other agents (Kim et al., 2003; Almenara et al.,

Abbreviations: GBM, glioblastoma multiforme; TMZ, temozolomide; MGMT, O^6 methylguanine- DNA methyltransferase; HDAC, histone deacetylase; HDACI, HDAC inhibitor; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; VPA, valproic acid; ROS, reactive oxygen species; NaB, sodium butyrate; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid

* Corresponding authors at: Department of Biochemistry, National Defense Medical Center, Taipei City 114, Taiwan, Republic of China.
E-mail addresses: hondy2195@yahoo.com.tw (D.-Y. Hueng), shihming@ndmctsgh.edu.tw (S.-M. Huang).

http://dx.doi.org/10.1016/j.biocel.2017.10.003
Received 8 June 2017; Received in revised form 3 October 2017; Accepted 6 October 2017
Available online 07 October 2017
1357-2725/

2002). To date, HDACIs can be divided into several structural classes, including hydroxamates, cyclic peptides, aliphatic acids and benzamides (Miller et al., 2003). More than 80 different HDACIs and analogs have been previously published or reviewed (Acharya et al., 2005). Potential HDACIs are still undergoing characterization, such as the hypercholesterolemia-lowering drugs, statins, or 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors. Recent studies have demonstrated that statins bind and inhibit HDAC activity, and then increase acetylated histone H3 accumulation and p21 expression level in human cancer cells (Lin et al., 2008).

As a prelude to this study, several HDACIs and statins were analyzed to investigate their cytotoxic effects in GBM cells. Based on the BBB permeability property of VPA and the efficacy of fluvastatin in our screening processes, these two drugs were chosen for the investigation of combinatory effects. The aim of our study was to examine whether synergistic effects can be achieved by combined application of VPA and fluvastatin. To this end, we examined under combined application the effects on apoptosis, double-strand DNA breaks, acetylation levels of histones H3 and H4 and expression levels of p21 and p53. Furthermore, the effects on the cell cycle and proliferation were examined.

2. Methods

2.1. Cell culture and reagents

The human glioblastoma multiforme cell line GBM8401 was grown in DMEM medium supplemented with 10% fetal bovine serum, Penicillin 100 units/ml, and Streptomycin 100 μg/ml. All cell lines were incubated at 37 °C in 5% CO_2. The HDACIs: VPA, sodium butyrate (NaB) and Trichostatin A (TSA) were purchased from Sigma-Aldrich; suberoylanilide hydroxamic acid (SAHA) and MS-275 were purchased from Luminescence Technology; LBH589 was kindly provided by the laboratory of Dr. Tai-Lung Cha (National Defense Medical Center, Taipei, Taiwan, ROC); fluvastatin, lovastatin and BEZ235 were purchased from Cayman Chemical Company. VPA was dissolved in H_2O; NaB, SAHA, LBH589, MS-275 and BEZ235 in dimethylsulfoxide (DMSO); TSA, fluvastatin and lovastatin in ethanol.

2.2. Immunoblot analysis

Cell lysates were prepared in lysis buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, and 1% Triton 100) at 4 °C. Proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, USA) and detected using antibodies against alpha-actinin (ACTN), p53, p21, B-Raf, Ac-H3 (acetylated form of histone H3 at lysines 9/14) and Ac-H4 (acetylated form of histone H4 at Serine 1|Lysine 5|Lysine 8|Lysine 12) (Santa Cruz Biotechnology, USA), γ-H2AX (phosphorylated form of H2AX at serine 139) (Epitomics, USA), cleaved poly(ADP-ribose) polymerase (cPARP), LC3 (light chain 3), mTOR, *p*-mTOR (phosphorylated form of mTOR at serine 2448), Akt, *p*-Akt (phosphorylated form of Akt at serine 473) and *p*-B-Raf (phosphorylated form of B-Raf at serine 445) (Cell signaling, USA).

2.3. RT-PCR analysis

RNA was extracted using total RNA reagent (Bioman, Taiwan, ROC). Then, 1.0 μg of total RNA was subjected to reverse transcription using MMLV Reverse Transcriptase (Epicentre Biotechnologies, USA) according to the manufacturer's instructions. The PCR forward and reverse primers used were 5′-GATGAAGCTCCCAGAATGCCAGAG-3′ and 5′-GAGTTCCAAGGCCTCATTCAGCTC-3′; 5′-CTGAGCCGCGACTGTGATGCG-3′ and 5′-GGTCTGCCGCCGTTTTCGACC-3′; and 5′-CTTCATTGACCTCAACTAC-3′ and 5′-GCCATCCACAGTCTTCTG-3′ for p53, p21, and GAPDH, respectively. The PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with UV light after ethidium bromide staining.

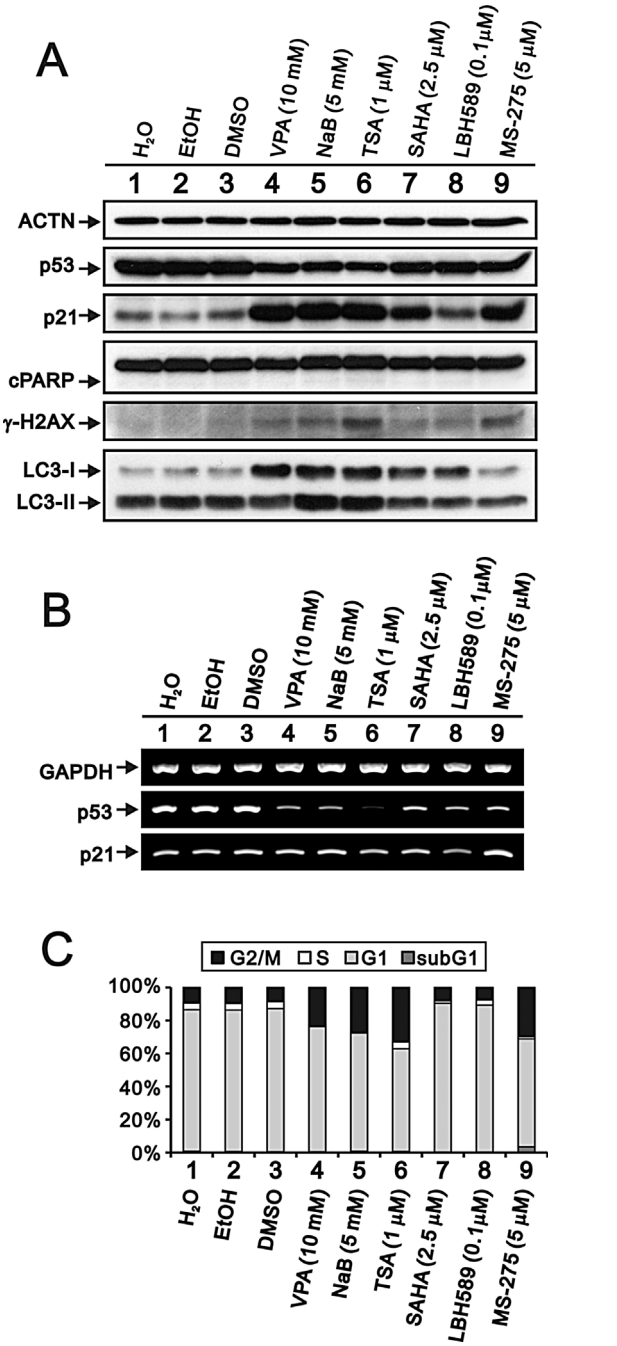

Fig. 1. The effect of HDACIs on cell cycle-related proteins and cell cycle profile. GBM8401 cells were treated with six different HDACIs. (A) After 24 h, the total cell lysates were subjected to immunoblot analysis with antibodies against p53, p21, cPARP, γ-H2AX and LC3. ACTN was the loading control. (B) After 24 h, p53 and p21 mRNA expressions were analyzed by RT-PCR. GAPDH served as the internal control. (C) After 48 h, the cell cycle profiles were determined by flow cytometry. Results are representative of three independent experiments and were compared to vehicle control (VPA was dissolved in H_2O; NaB, SAHA, LBH589 and MS-275 in DMSO; and TSA in ethanol).

2.4. Flow cytometry analysis of proliferation, cell cycle and apoptosis

For cell cycle analysis, the distribution was determined by measuring DNA content using fluorescence activated cell sorting (FACS). Cells were fixed in 70% ice-cold ethanol and kept at −20 °C overnight. Before analysis, the harvested cells were washed with ice-cold PBS twice and stained with propidium iodide (PI) solution (5 μg/ml PI in PBS, 0.5% Triton X-100 and 0.5 μg/ml RNase A) for 30 min at 37 °C in the dark.