Tzu Chi Medical Journal 28 (2016) 103–108

Contents lists available at ScienceDirect

Tzu Chi Medical Journal

journal homepage: www.tzuchimedjnl.com

Original Article

Effects of caffeine on cell viability and activity of histone deacetylase 1 and histone acetyltransferase in glioma cells

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ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form
10 May 2016
Accepted 3 June 2016
Available online 1 August 2016

Keywords:
Caffeine
Glioma cells
Histone acetyltransferase
Histone deacetylase 1
p300

ABSTRACT

Objective: The prognosis of patients with glioblastoma remains poor even after various treatments such as surgery, radiotherapy, and chemotherapy. Thus, development of new drugs is urgently needed. The mechanisms underlying the cytotoxicity of caffeine in glioma cells are not clearly understood. This study aimed to assess the activities of histone deacetylase 1 (HDAC1) and histone acetyltransferase (p300) in RT2 glioma cells treated with caffeine.

Materials and Methods: Cell viability and activity of HDAC1 and p300 in RT2 glioma cells were assayed after treatment with caffeine for 48 hours.

Results: Cell viability decreased significantly after treatment with 0.5mM, 1mM, and 2mM caffeine. HDAC1 protein activity decreased significantly with various concentrations of caffeine, whereas the activity of p300 increased significantly. In addition, the viability of RT2 cells remained high, but HDAC1 activity decreased, and p300 activity increased markedly with 0.5mM caffeine treatment. We used microRNA and small interfering RNA (siRNA) to regulate HDAC1 and p300 to further understand the impact on glioblastomas. siRNA downregulated p300 and thus increased the viability of RT2 cells, therefore, caffeine combined with siRNA abolished the efficacy of caffeine, which confirmed that caffeine upregulated p300 and reduced cell viability. We also found increased HDAC1 activity when RT2 cells were treated with a combination of caffeine and miR-449a and thus increased the viability of RT2 cells.

Conclusion: Our data suggest that a new strategy, caffeine, could increase glioma cell death by decreasing HDAC1 activity and/or by increasing p300 activity. The changes in HDAC1 and p300 activities appeared to occur earlier than loss of RT2 cells.

1. Introduction

Malignant glioma is the most common primary brain tumor in adults. The poor prognosis of malignant glioma is largely due to a highly deregulated tumor genome with opportunistic deletion of tumor suppressor genes, amplification and/or mutational hyperactivation of receptor tyrosine kinase, and defects in the apoptosis signaling machinery [1]. In addition, abnormal expression of specific genes is associated with malignant glioma [2–5].

Epidemiological studies have also shown that consumption of caffeinated beverages, including coffee and tea, may reduce the risk of adult glioma [6,7]. *In vitro*, caffeine (2mM) enhances the cytotoxicity of cisplatin and camptothecin in human U251 glioma cell lines [8]. Caffeine blocks glioblastoma invasion and extended survival [9] and increased caspase-dependent apoptosis of glioma cells [10]. Our previous studies showed that tetrandrine and caffeine (0.5mM and 1mM) could induce glioma cell death [11]. Caffeine reduces glioma cell proliferation through G0/G1-phase cell cycle arrest by suppressing retinoblastoma protein phosphorylation [10]. Caffeine can attenuate G2 delay and enhance the cytotoxicity of

Conflict of interest: none.

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http://dx.doi.org/10.1016/j.tcmj.2016.06.005

cisplatin and camptothecin in human brain tumor cell lines [8]. Caffeine can inhibit some kinase activities, including various forms of mechanistic target of rapamycin and phosphoinositide 3-kinase (PI3K) in tumor cells [12]. Also, caffeine can increase phosphatase and tensin homolog (PTEN) expression, leading to PI3K–protein kinase B (Akt) pathway inactivation, and blocking of osteosarcoma cell proliferation [13]. Caffeine confers radiosensitization of PTEN-deficient malignant glioma cells by enhancing ionizing-radiation-induced G1 arrest and negatively regulating Akt phosphorylation [14].

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are involved in determining the state of acetylation of histones, which are the early steps for genetic transcription [2]. There are reports that altered activities of HDACs and HATs are associated with cancer formation. For example, overexpression of HDAC1 is associated with human gastric and prostate cancers, and with malignant glioma [3]. The antiproliferative effect of HDAC inhibitors is mediated by G0/G1 cell cycle arrest [15]. HDAC inhibitor activates p53 transcription and acetylation promotes upregulated modulator of apoptosis to catalyze the mitochondrial pathway to induce growth inhibition, cell cycle arrest, and apoptosis [16]. Thus, HDAC1 has recently been recognized as a promising target for cancer therapy, including treatment of malignant glioma. In addition, decreased expression of HATs is associated with several diseases, such as cardiac hypertrophy, asthma and cancer [4]. Loss of heterozygosity of the HAT p300 was found in 80% of malignant gliomas [5]. Loss of microRNA (miR)-449a, an endogenous HDAC-1 inhibitor, may promote abnormal performance of HDAC-1 in prostate cancer, contributing to the cause of the disease [17]. The effects of caffeine on the activity of HDACs and HATs in glioma cells are still unknown. The detailed effects and mechanisms underlying caffeine-induced cytotoxicity in glioma cells remain unclear.

In this study, we investigated the effects of caffeine on the viability of malignant glioma cells and the activities of HDAC1 and p300. We hypothesized that caffeine could increase death of RT2 glioma cells by decreasing HDAC1 activity and increasing p300 activity.

2. Materials and methods

2.1. Cell line

RT2 cells were derived from an avian-sarcoma-virus-induced brain tumor in a Fischer 344 rat. This cell line was provided by National Taiwan University Hospital [18], and was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2mM L-glutamate and 100 U/mL penicillin at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

2.2. siRNA and microRNA transfection

Control small interfering RNA (siRNA) (Thermo Scientific siGLO siGENOME, and ON-TARGET*plus* Control Reagents), siRNA against p300 (Thermo Scientific Dharmacon siGENOME and ON-TARGET*plus* siRNA Reagents), microRNA (miRNA) mimic and miR-449a (miRNA for HDAC1, Thermo Scientific miRIDIAN microRNA Mimics, Hairpin Inhibitors and Negative Controls) were obtained from Dharmacon (Franklin Lakes, NJ, USA). Cells (1×10^4) were seeded on 12-well plates the day before transfection. The medium was switched to Dulbecco's Modified Eagle's Medium and either control siRNA or anti-p300 siRNA in Oligofectamine was added to the culture medium for 48 hours (final concentration 25 nmol/L).

2.3. Cell viability assay

Cell viability was measured 48 hours after treatment with various concentrations of caffeine (0mM, 0.5mM, 1mM, and 2mM) by a colorimetric assay for 96-well plates using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1; Clontech, Palo Alto, CA, USA). Each plate contained blanks, controls, and treatment groups. Treatment consisted of addition of 10 μL of premixed WST-1 cell proliferation reagent to each well (1:10 final dilution).

The plate was incubated for 4 hours at 37°C in a humidified atmosphere maintained at 5% CO_2. Absorbance at 450 nm (reference wavelength 690 nm) was measured using a multiwell plate reader (Anthos Zenyth 3100 Microplate Multimode Detector; Salzburg, Austria).

2.4. HDAC1 activity assay

Assays were performed using the enzyme-linked immunosorbent assay kit for HDAC1 (USCN Life Science, Wuhan, China). Briefly, 100 μL of cells or standards were added to each well and incubated for 2 hours at 37°C. Then, the liquid was removed from each well. We added 100 μL Detection Reagent A to each well and incubated for 1 hour at 37°C. The liquid was removed from each well and the wells were washed with wash solution three times. Detection Reagent B (100 μL) was added to the wells and incubated for 30 minutes at 37°C. The liquid was removed from each well and the wells were washed with wash solution five times. Substrate solution (90 μL) was added followed by incubation for 20 minutes at 37°C. Stop solution (50 μL) was added and the plates were read on a multiwell plate reader at 450 nm. HDAC1 activity was expressed as the relative optical density (OD) values per nanogram of protein sample.

2.5. HAT (p300) activity assay

Assays were performed using the HAT activity colorimetric assay (BioVision Research Products, Mountain View, CA, USA). Briefly, 50 μg purified proteins from cells were diluted in 40 μL deionized distilled water; 68 μL assay mix was added followed by incubation at 37°C for 1–4 hours. Samples were read in an enzyme-linked immunosorbent assay plate reader at 440 nm. HAT (p300) activity was expressed as relative OD values per microgram of protein sample.

2.6. Statistical analysis

The data are presented as the mean ± standard deviation, unless indicated otherwise. The expression levels of HDAC1 or p300 were compared separately among all groups by one-way analysis of variance with *post hoc* Bonferroni correction. All analyses were performed by commercial software (Stata Corp LP, College Station, TX, USA), and p values < 0.05 were considered statistically significant. Data are representative of three independent experiments (error bars, standard deviation of triplicate samples).

3. Results

Fig. 1 shows the effects of various concentrations of caffeine (0mM, 0.5mM, 1mM, and 2mM) on the viability of RT2 glioma cells at 48 hours. Compared with the control group without caffeine treatment (100%), the viability decreased significantly when RT2 cells were treated with 0.5mM (67.3 ± 1.16%), 1mM (54.5 ± 2.53%), or 2mM (10.3 ± 1.08) caffeine ($p < 0.0001$ vs. control group).

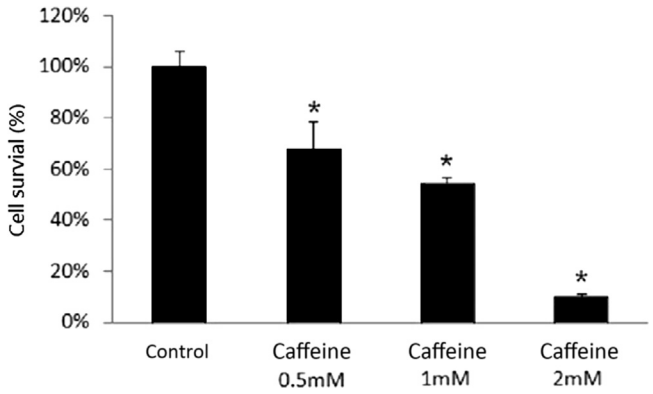

Fig. 1. Effects of caffeine on the viability of RT2 glioma cells at 48 hours. Compared with the control group without caffeine treatment (100%), viability decreased significantly when RT2 cells were treated with 0.5mM or more caffeine. $^{*}p < 0.0001$ versus control group.

Fig. 2 shows the effects of various concentrations of caffeine or caffeine combined with miR-449a on the viability of RT2 glioma cells at 48 hours. Compared with the negative control group (100%), the viability was unchanged when cells were treated with miR-449a (99.3 ± 1.56%) only. miRNA did not affect RT2 cell growth. In contrast, the caffeine-decreased viability effect was abolished when RT2 cells were treated with a combination of miRNA and 0.5mM (82.9 ± 2.45%), 1mM (63.6 ± 1.15%), or 2mM (25.5 ± 1.95%) caffeine ($p < 0.0001$ vs. caffeine only).

Fig. 3 shows the effects of various concentrations of caffeine or caffeine combined with p300 siRNA on the viability of RT2 glioma cells at 48 hours. Compared with the negative control group (100%), the viability was unchanged when cells were treated with siRNA (107.3 ± 3.07%) only. siRNA did not affect RT2 cell growth. In contrast, the caffeine-decreased viability effect was abolished when RT2 cells were treated with a combination of siRNA and 0.5mM (96.4 ± 2.95%), 1mM (59.7 ± 2.02%) or 2mM (23.6 ± 1.36%) caffeine ($p < 0.0001$ vs. caffeine only).

Fig. 4 shows the effects of various concentrations of caffeine or caffeine combined with miR-449a on the activity of HDAC1 in RT2 glioma cells at 48 hours. Compared with the negative control group (1.17 ± 0.11 ng/mL), the activity of HDAC1 showed no significant changes when RT2 cells were treated with miR-449a (1.04 ± 0.15 ng/mL) only. The activity of HDAC1 decreased significantly when RT-2 cells were treated with 0.5mM (0.43 ± 0.20 ng/mL), 1mM (0.39 ± 0.19 ng/mL) or 2mM (0.20 ± 0.02 ng/mL) caffeine ($p < 0.0001$vs. control group). Activity of HDAC1 increased significantly when cells were treated with a combination of miRNA-449a and 0.5mM (1.06 ± 0.25 ng/mL), 1mM (0.86 ± 0.23 ng/mL) or 2mM (0.5 ± 0.02 ng/mL) caffeine ($p < 0.0001$ vs. caffeine only).

Fig. 5 shows the effects of various concentrations of caffeine or caffeine combined with siRNA on the activity of p300 in RT2 glioma cells at 48 hours. Compared with the negative control group (0.45 ± 0.03, OD 440 nm), the activity of p300 showed no significant changes when RT2 cells were treated with siRNA (0.44 ± 0.04, OD 440 nm) only. The activity of p300 increased significantly when RT-2 cells were treated 0.5mM (0.70 ± 0.05, OD 440 nm), 1mM (0.78 ± 0.06, OD 440 nm) or 2mM (0.76 ± 0.06, OD 440 nm) caffeine ($p < 0.0004$ vs. control group). Furthermore, the activity of p300 decreased significantly when RT2 cells were treated with a combination of siRNA and 0.5mM (0.39 ± 0.01, OD 440 nm), 1mM (0.38 ± 0.01, OD 440 nm) or 2mM (0.37 ± 0.07, OD 440 nm) caffeine. There were no significant differences compared with the control group.

4. Discussion

Our previous studies showed that tetrandrine and caffeine (0.5mM and 1mM) induce glioma cell death, possibly *via* increasing eukaryotic initiation factor-2α phosphorylation, decreasing cyclin-D1 expression, and increasing the caspase-dependent and -independent apoptosis pathways [11]. The present study showed that caffeine increased glioma cell death, decreased HDAC1 activity, and increased p300 activity; in addition, we found that the changes in HDAC1 and p300 activities appeared to occur earlier than loss of RT2 cells. In other words, the viability of RT2 cells remained high, but HDAC1 decreased and p300 increased markedly with 0.5mM caffeine treatment. We also found that HDAC1 activity was reversed

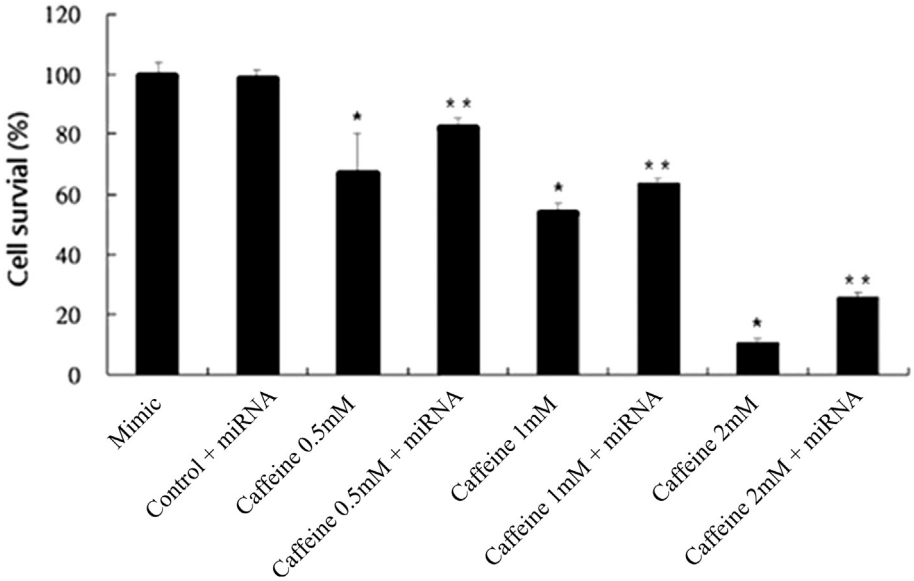

Fig. 2. RT2 cells transfected with miR-449a and treated with caffeine at 48 hours. Compared with the negative control group (100%), viability decreased significantly when RT2 cells were treated with 0.5mM, 1mM, or 2mM caffeine. In contrast, the caffeine-decreased viability effect was abolished when RT2 cells were treated with a combination of microRNA and caffeine. $^{*}p < 0.0001$ versus control group, $^{**}p < 0.0001$ versus caffeine only. miRNA = microRNA.

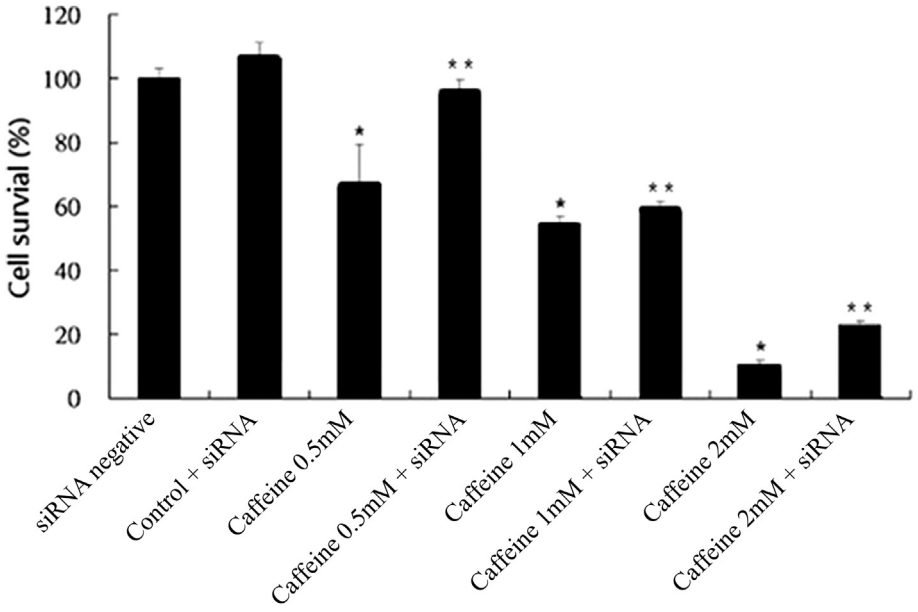

Fig. 3. RT2 cells transfected with p300 siRNA to silence its expression at 48 hours. Compared with the negative control group (100%), viability decreased significantly when RT2 cells were treated with 0.5mM, 1mM, or 2mM caffeine. In contrast, caffeine-decreased viability effect was abolished when RT2 cells were treated with a combination of microRNA and caffeine. $^{*}p < 0.0001$ versus control group, $^{**}p < 0.0001$ versus caffeine only. siRNA = small interfering RNA.

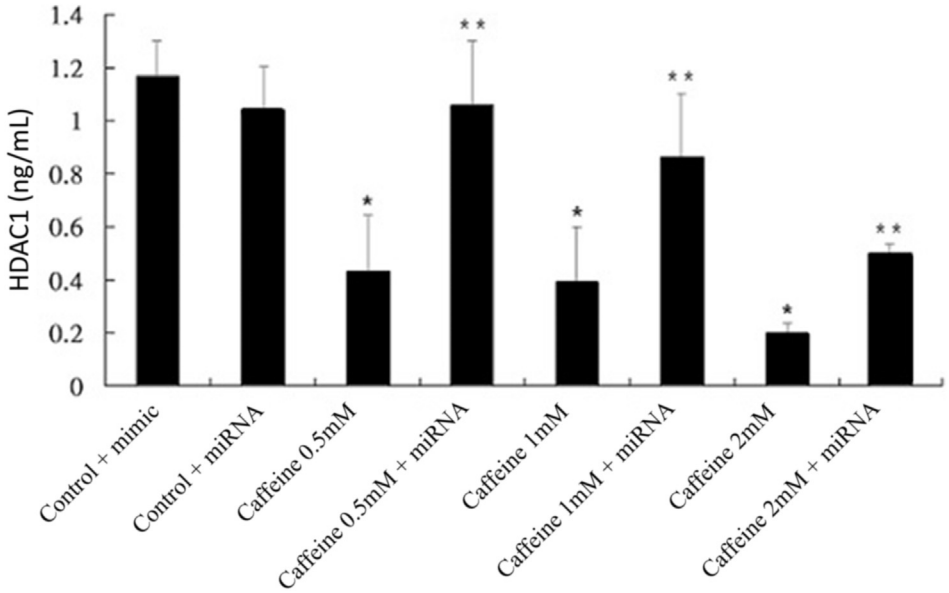

Fig. 4. Effects of various concentrations of caffeine or caffeine combined with miR-449a on activity of HDAC1 in RT2 glioma cells at 48 hours. Compared with the control group without caffeine treatment, expression of HDAC1 decreased significantly when RT2 cells were treated with 0.5 mM or more caffeine. The activity of HDAC1 increased significantly when RT2 cells were treated with a combination of miR-449a and various concentrations of caffeine. $^{*}p < 0.0001$ versus control group, $^{**}p < 0.0001$ versus caffeine only. HDAC = histone deacetylase; miRNA = microRNA.

when RT2 cells were treated with a combination of caffeine and miR-449a.

Both HDACs and HATs are involved in many physiological and pathological processes by the modulation of transcription of many genes. HDAC1 is a predominantly nuclear protein with ubiquitous expression; it is induced upon growth factor activation and is increased in highly proliferative tissues, embryonic stem cells, several transformed cell lines, and in the majority of cancers [19,20]. Consistent with this, HDAC inhibitors might induce p53-dependent and p53-independent Bax-mediated neuronal apoptosis [21]. HDAC inhibitor is associated with improved overall and progression-free survival in glioblastoma but worse outcomes in Grade II/III gliomas treated with temozolomide [22].

HATs are also essential for cell proliferation, differentiation, and apoptosis [23]. Decreased expression or mutations of HATs are associated with several types of cancer [4,5]. Previous studies showed that mitogen-activated protein kinases phosphorylate several types of HATs and directly increase their enzymatic activities [24,25], or regulate their activity indirectly by modifying signaling pathways affecting HAT activity [26,27].

Imbalanced or altered expression or activities of HDACs and HATs are associated with cancer formation. Previous reports reveal

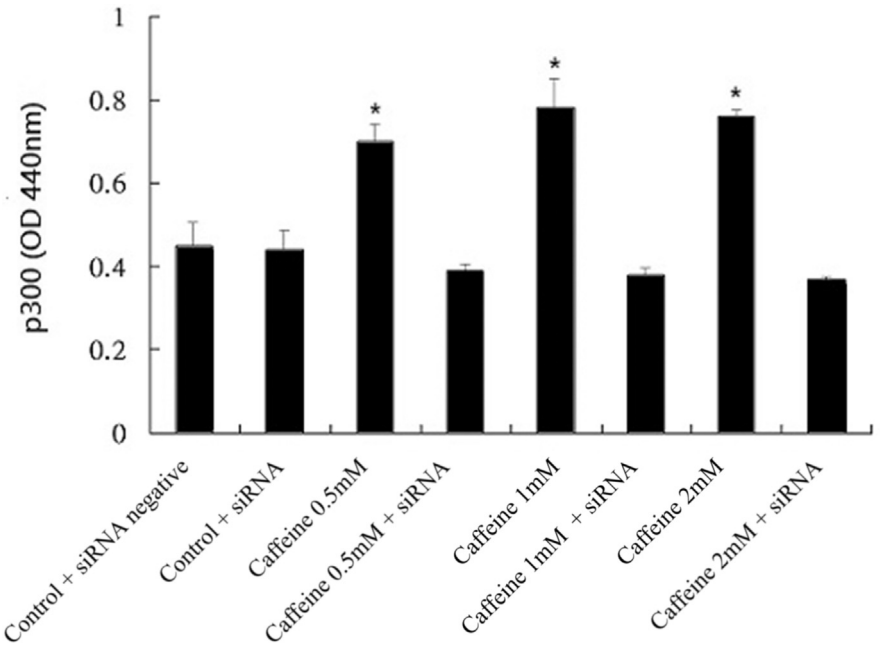

Fig. 5. Effects of various concentrations of caffeine or caffeine combined with siRNA on the activity of p300 in RT2 glioma cells at 48 hours. Compared with the control group without caffeine treatment, expression of p300 increased significantly when RT2 cells were treated with 0.5 mM or more caffeine. Activity of p300 decreased significantly when RT2 cells were treated with a combination of siRNA and various concentrations of caffeine. $^{*}p < 0.0004$ versus control group. siRNA = small interfering RNA.

that overexpression of HDAC1 is associated with malignant glioma [3], whereas loss of heterozygosity of the HAT p300 was found in 80% of malignant gliomas [5]. In this study, we found that caffeine modulated the imbalance of HDAC1 and p300 in RT2 cells by decreasing HDAC1 activity and increasing p300 activity. These effects might account for caffeine-induced cytotoxicity of RT2 cells.

In conclusion, our data suggest that a new strategy, caffeine, could increase glioma cell death; possibly by decreasing HDAC1 activity and/or increasing p300 activity. In addition to traditional chemotherapy, caffeine might be added as a complementary treatment for human gliomas in the future. Further clinical trials are warranted to document this cell study.

Acknowledgments

This work was supported by a grant from Buddhist Dalin Tzu Chi General Hospital (DTCRD100(2)-I-06) in Chiayi, Taiwan.

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