Letter to the Editor

Valproic Acid Enhances the Anti-tumor Effect of (-)-gossypol to Burkitt Lymphoma Namalwa Cells*

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Burkitt lymphoma is a highly aggressive B-cell neoplasm. New therapeutic methods are needed to overcome the adverse effect of intensive chemotherapy regimens. Valproic acid and (-)-gossypol are two kinds of chemical compounds used as new anti-tumor drugs in recent years. To investigate the anti-tumor effect of valproic acid and (-)-gossypol, Burkitt lymphoma Namalwa cells were cultured and treated with valproic acid and (-)-gossypol at different concentrations. The proliferation of Namalwa cells was dramatically suppressed after the combination treatment with 2 mmol/L valproic acid and 5 μmol/L (-)-gossypol. The combined treatment also enhanced intrinsic apoptosis by down-regulating anti-apoptotic protein Mcl-1. Moreover, the autophagy flux significantly increased in Namalwa cells after combined treatment. However, the enhanced autophagy showed little effect on cell survival with present regimen. The results confirmed that combination of valproic acid and (-)-gossypol had synergistic anti-tumor effect to Burkitt lymphoma Namalwa cells. The related mechanisms might include the down-regulation of anti-apoptotic protein Mcl-1 and avianized pro-survival role of autophagy.

Burkitt lymphoma is a highly malignant B-cell non-Hodgkin lymphoma. Various therapeutic strategies have been developed to treat this disease, but the effects are quite different among Burkitt lymphoma patients. In recent years, a typical anti-epileptic drug, Valproic acid (VPA) shows effective anti-tumor effect *in vitro* and *in vivo*[1]. It has been confirmed that VPA alone can induce cell apoptosis, stimulate cell differentiation and suppress cell proliferation in the treatment of multi tumors. In addition, VPA showed dramatic synergistic effects with some anti-tumor drugs to prostate cancer cells[2]. It has been confirmed that VPA enhance bortezomib-mediated cell proliferation inhibition and apoptosis induction to acute myeloid leukemia cells. For B-cell lymphomas, VPA augmented the anti-tumor effect of Rituximab *via* increasing CD20 expression.

(-)-gossypol, a metabolite from cotton seeds, which is used for male contraception in clinical practice, can also kill tumor cells *in vitro* and *in vivo*[3]. In chronic lymphocytic leukemia cells, (-)-gossypol induces cell apoptosis *via* activation of BAX and release of cytochrome c and apoptosis-inducing factor[4]. Furthermore, (-)-gossypol could overcome traditional chemotherapeutic resistance in Jurkat T leukemia cell overexpressing Bcl-2 and enhance the cytotoxicity of imatinib in human chronic myeloid leukemic cells.

Our previous study has confirmed the anti-tumor effect of (-)-gossypol to Burkitt lymphoma Namalwa cells[5]. However, as effective anti-tumor drugs, VPA and (-)-gossypo has not been used in a combined formula to treat Burkitt lymphoma before. In this study we investigated the possible synergistic anti-tumor effect of VPA and (-)-gossypol to Namalwa cells and the related mechanisms to provide experimental evidence for its translational clinical application.

Human Burkitt lymphoma Namalwa cell line (American Type Culture Collection) was cultured in RPMI 1640 medium (Hyclone, USA) with 10% fetal bovine serum (Hyclone, USA), streptomycin (100 μg/mL) and penicillin (100 U/mL) (Beyotime, China)

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at 37 °C in 5% CO_2 humidied incubator. VPA, (-)-gossypol, CQ were from Sigma-Aldrich (Louis, MO, USA). Cell Counting Kit-8 was from Dojindo (Dojindo, Japan). Immunoblotting Polyvinylidene fluoride (PVDF) membrane was from Bio-Rad (Hercules, CA). West Dura Extended Duration Substrates were from Thermo Scientific (Massachusetts, USA). Bradford protein assay kit, caspase 3 Activity Assay Kit and Cell Mitochondria Isolation Kit were from Beyotime (Beijing, China). CCK-8 assay for cell proliferation was carried out as described previously. Briefly, Namalwa cells were seeded into 96-well plate with around 2000 cells/well and cultured overnight. Then the cells were given different treatments for 36 h. At last, the cells were treated with CCK-8 reagents for OD450 detection with microplate reader.

The mitochondria were separated according to the manufacturer's protocol (Beyotime, Beijing, China). Briefly, the treated cells were suspended by using mitochondrial separation reagent and placed on ice for 15 min. The mixture was ground for twenty times, then the mixture was centrifuged at 600 g for 10 min and the supernatant was collected. Subsequently, the supernatant was centrifuged at 11,000 g for 10 min. The precipitant is the mitochondrial extracts, while the supernatant is cytosolic extracts without mitochondria.

The total proteins of the treated cells were extracted from lysate and quantified by Bradford protein assay kit. Then the proteins were separated by SDS-PAGE before transferring onto PVDF membrane (Bio-Rad, Hercules, CA, USA) and detected by using immunoblotting. Primary antibodies of LC3I/II was from Cell Signaling Technology (Boston, MA, USA), Bcl-2, Bcl-xl, Mcl-1 and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), cytochrome c and COX IV were from Sigma-Aldrich (Louis, MO, USA), Ac-H3 was from bioss (Beijing, China). Horseradish Peroxidase-conjugated anti-mouse/rabbit IgG was from Pierce (Rockford, IL, USA).

He cells were stained with Trypan blue solution according to the manufacturer's instructions. After counting living cells and dead cells with microscope, the total death rate (%) was calculated.

All the data were presented as the mean±SD from at least three independent experiments. The difference between groups was determined by using one-way ANOVA with the post hoc Tukey test. *P*-value <0.05 represents a statistical significant difference.

In this study, Namalwa cells were treated with VPA at different concentrations for 36 h and then CCK-8 reagents were used to calculate the cell survival rate. As shown in Figure 1A, 0.5 mmol/L, 1 mmol/L, and 2 mmol/L VPA had little effect on cell proliferation, while 5 mmol/L and 10 mmol/L VPA significantly suppressed cell proliferation, suggesting that VPA alone had cytotoxicity to Namalwa cells. To study the effect of VPA combined with (-)-gossypol, Namalwa cells were treated with 5 μmol/L (-)-gossypol combined with or without 2 mmol/L VPA. Combination treatment had significant proliferation inhibition than (-)-gossypol or VPA alone did (Figure 1B). In addition，the analysis report of CCK-8 assay using CalcuSyn software also verified the effective synergistic effect in the combination of (-)-gossypol and VPA. Taken together, the combination of VPA and (-)-gossypol enhanced the anti-tumor effect to Burkitt lymphoma Namalwa cells.

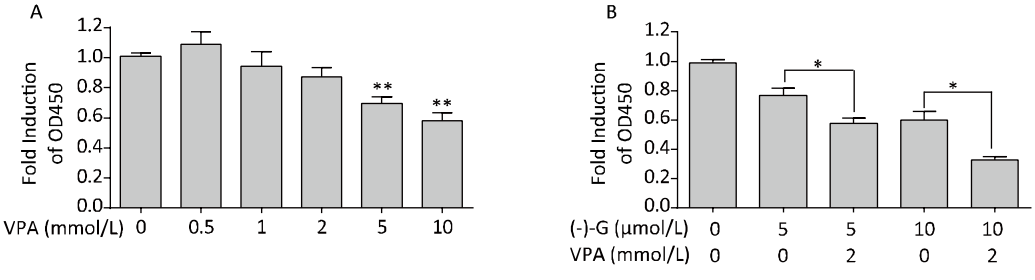

Figure 1. The combined treatment of VPA and (-)-gossypol significantly inhibited cell proliferation. (A) Namalwa cells were seeded in a 96-well plate and cultured overnight. Then the cells were treated with VPA at different concentrations for 36 h. Subsequently, CCK-8 reagents were added and the value of OD450 were recorded after 60 min incubation. Data were obtained from three independent experiments and presented as mean±SD. **, P<0.01 *vs.* Control group. (B) Namalwa cells were treated with 5 μmol/L or 10 μmol/L (-)-gossypol alone or a combination of (-)-gossypol and 2 mmol/L VPA for 36 h. After treatment, cck-8 assay was used for the assessment of cell proliferation. Data were expressed as mean±SD. **, P<0.01; *, P<0.05.

To further study the mechanisms of combination treatment-mediated cytotoxicity, the intrinsic apoptosis was determined. As shown in Figure 2A, the combination treatment markedly promoted the translocation of cytochrome c from mitochondria to cytoplasm, which is a key step in intrinsic apoptosis. The combination treatment also obviously enhanced the activity of caspase 3 protease and subsequent cleavage of caspase 3 (Figure 2B and 2C). It has been reported that intrinsic apoptosis is tightly regulated by anti-apoptotic proteins, such as Bcl-2 and Mcl-1. As shown in Figure 2C, Bcl-2 protein level did not show obvious change in Namalwa cells with either single drug treatment or combination treatment. Significantly, the level of Mcl-1 in Namalwa cells was down-regulated by (-)-gossypol treatment and showed more remarkable decrease after the combination treatment of VPA and (-)-gossypol. Mcl-1, myeloid cell leukemia-1, is an anti-apoptotic member of Bcl-2 family. Mcl-1 maintains mitochondrial integrity and protects cells from intrinsic apoptosis under stress *via* binding to pro-apoptotic proteins, such as Bax, Bak and Bim[6]. Due to the important role of Mcl-1 in Bcl-2 inhibitor-induced apoptosis[7], we deduced that the down-regulation of Mcl-1 by the combination of VPA and (-)-gossypol might be a momentous factor to the augmentation of intrinsic apoptosis. These results indicated that the augmentation of intrinsic apoptosis could be a main factor for the anti-tumor effect of the combination treatment.

Autophagy is a self-protection process for cell survival under stress. Multiple evidence confirmed that autophagy was closely related to cancer therapy. On one hand, autophagy induced by anti-tumor treatments prevents cells from apoptosis and promotes cell survival. On the other hand, over-activated autophagy could lead to cell death. It has been reported that (-)-gossypol induced cytoprotective autophagy in human Burkitt lymphoma Namalwa cells[8], while VPA can induce autophagic cell death in human glioma cell lines[9]. Therefore, the role of combination treatment induced

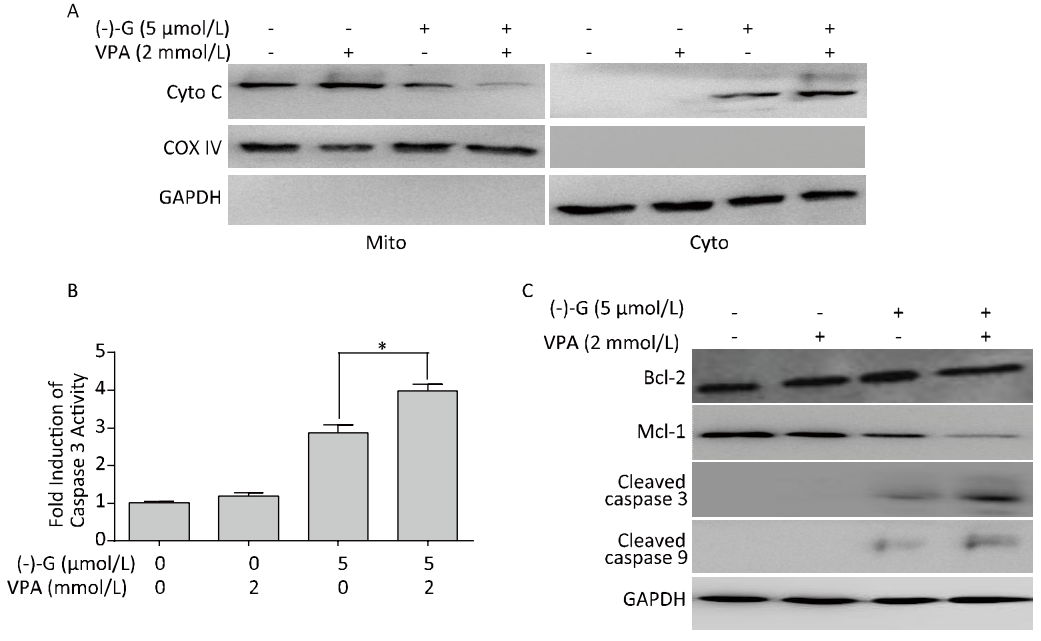

Figure 2. The combination of VPA and (-)-gossypol enhanced intrinsic apoptosis. Namalwa cells were treated with 5 μmol/L (-)-gossypol in the presence of 2 mmol/L VPA or not for 24 h. Then (A) Mitochondrial protein and cytoplasmic protein were separately extracted and loaded into gels of SDS-PAGE for immunoblotting. Here COX IV and GAPDH were separately regarded as mitochondrial protein and cytoplasmic protein internal control. (B) The caspase 3 activity of treated cells were evaluated by caspase 3 detection assay according to the manufacturer's protocol. Data were obtained from three independent experiments and presented as mean±SD. *, P<0.01. (C) The total protein of cells were obtained and the protein level of Bcl-2, Mcl-1, cleaved caspase 3, and caspase 9 were measured by immunoblotting.

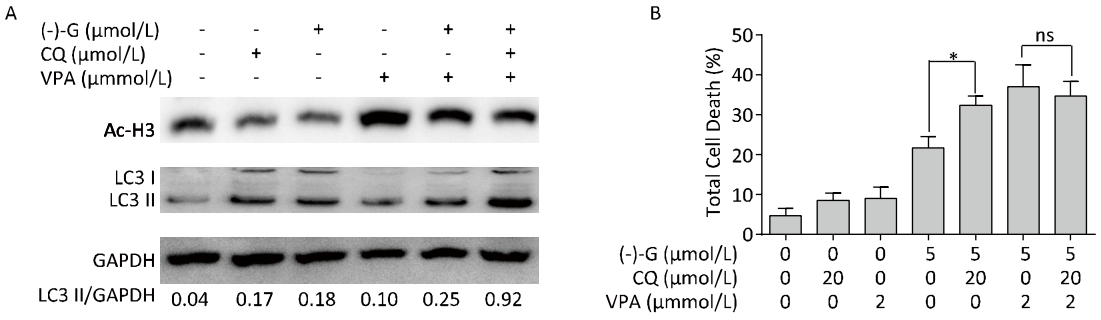

Figure 3. The level of autophagy increased in cells co-treated with VPA and (-)-gossypol. (A) Namalwa cells were treated with 2 mmol/L VPA or 5 μmol/L (-)-gossypol respectively or in combination with 20 μmol/L CQ for 24 h. Then the protein level of Ac-H3 and LC3I/II were measured by immunoblotting. (B) Cells were treated as indicated above for 36 h. Subsequently, Trypan blue exclusion assay was used to estimate the total death rate. Data were obtained from three independent experiments and presented as mean±SD. *, $P<0.01$; ns means no significance.

autophagy in cell survival and cell death has been great interesting. As shown in Figure 3A, the combination treatment of VPA and (-)-gossypol significantly increased the protein level of LC3II. Meanwhile, autophagy inhibitor CQ which can block the fusion of autophagosome and lysosome which further promoted the combination treatment-mediated LC3II accumulation. LC3II is a critical component of autophagosome which can be considered as a marker of autophagy. These data showed that VPA can obviously enhanced (-)-gossypol-induced autophagy. We further investigated the role of autophagy in cells treated with a combination of VPA and (-)-gossypol. As shown in Figure 3B, CQ dramatically enhanced (-)-gossypol-induced cytotoxicity, but had little effect on the cytotoxicity triggered by a combination VPA and (-)-gossypol. These results indicated that VPA might participate in regulating the level of (-)-gossypol-induced autophagy, which decrease the cytotoxicity induced by CQ. Acetylation could influence the pro-survival or pro-death role of autophagy[10]. To test if the combination of drugs altered the inhibitory effect of VPA on histone deacetylase, the acetylation level of histone 3 was examined. It was confirmed that VPA-treated cells maintained a high acetylation status with or without addition of (-)-gossypol (Figure 3A). As VPA can inhibit the activity of histone deacetylase, we deduced that (-)-gossypol-mediated pro-surcival role of autophagy might be avianized when cells were under a hyperacetylation status induced by VPA treatment. The possible molecular mechanisms still need further study.

In summary, our results confirmed the efficacy of combined treatment of VPA and (-)-gossypol on Burkitt lymphoma Namalwa cells. In our study, the anti-apoptotic protein Mcl-1 was dramatically down-regulated after the combination treatment. Additionally, the combination of VPA and (-)-gossypol triggered an enhanced autophagic flux. Though the autophagy level dramatically increased, the autophagy inhibitor CQ didn't influence the anti-tumor effect of combined treatment of VPA and (-)-gossypol. Therefore, we deduce that the autophagy might be non-protective in the context of combined treatment. As VPA and (-)-gossypol are both oral-available and well-tolerated agents in clinical practice, this combination therapy may be feasible in clinical practice and a promising strategy for the treatment of aggressive lymphomas, such as Burkitt lymphoma.

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