Combined Valproic Acid and Celecoxib Treatment Induced Synergistic Cytotoxicity and Apoptosis in Neuroblastoma Cells

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Abstract. *Background: The effects of combined valproic acid (VPA) and celecoxib treatment on cancer cells have not been reported. In this study, we examined the effects of VPA and celecoxib, alone and combined, on human SH-SY5Y neuroblastoma cells. Materials and Methods: The cytotoxicity effects of VPA, celecoxib, and combined VPA and celecoxib treatment on neuroblastoma cells were studied. The apoptotic fraction and the cell cycle distribution of neuroblastoma cells were analyzed by flow-activated cell sorter analysis. Western blot analysis was used to investigate the expression of cyclooxygenase-2, p53, 14-3-3σ, p21, p27, Bcl-2 and Bax in neuroblastoma cells treated with various regimens. Results: Combined VPA and celecoxib treatment caused more cytotoxicity and apoptosis in neuroblastoma cells than individual drug treatment ($p<0.05$). In addition, combination treatment caused more neuroblastoma cells to accumulate in the G_0/G_1 phase of the cell cycle ($p<0.04$) and induced higher p21 and p27 expression than individual drug treatment or control. Conclusion: Combined VPA and celecoxib treatment induced more cytotoxicity and apoptosis in neuroblastoma cells than individual drug treatment. The effects were probably related to the increased p21 and p27 expression, and G_0/G_1 accumulation of neuroblastoma cells.*

Neuroblastoma is the most common solid malignancy in children (1). The prognosis of advanced neuroblastoma is poor, even using multimodality treatment (1). Because the currently used therapeutic regimens have limited efficacy and frequently cause serious side-effects, it is mandatory to develop an effective treatment.

Valproic acid (di-*n*-propylacetic acid, VPA) is a widely used drug for epilepsy (2). In addition to epilepsy, it also has antitumor effects (2). VPA inhibits proliferation and induces differentiation of malignant cells including leukemia, lymphoma, glioma, teratocarcinoma, medullo-blastoma and atypical teratoid/rhabdoid tumor cells and clinically it has been applied to the treatment of leukemia and some solid tumors (3-7). Further, VPA has also been found to suppress the proliferation of neuroblastoma cells (4, 8, 9). VPA has various actions including induction of apoptosis; activation of the expression of neural cell adhesion molecule, Bcl-2, and p73; reduction of the expression of n-Myc; increase of the expression of intracellular Notch-1 and Hes-1 and activation of the Notch signaling cascade; induction of cell cycle arrest in the G_0/G_1 phase; activation of the phosphorylation of Akt and glycogen synthase kinase-3β; stimulation of the inhibitor of angiogenesis such as thrombospondin-1 and activin A (8, 10, 11). Furthermore, it causes histone deacetylase (HDAC) inhibition, proteasomal degradation of HDAC, extracellular-regulated kinase activation, protein kinase C inhibition, Wnt-signaling activation, down-regulation of telomerase activity and DNA demethylation (2, 4, 12). All these actions might play a role in the anticancer effects of VPA; however the actual mechanisms remain unclear.

Cyclooxygenase (COX) is the rate-limiting enzyme catalyzing the conversion of arachidonic acids into prostaglandins, lipid mediators implicated in various physiological and pathophysiological processes (13). COX-2 is an isoform of COX. In normal tissue, COX-2 is either not expressed or expressed at a low level; on the other hand, increased expression of COX-2 is noted in various tumors and is closely associated with cancer progression and metastasis (14). Celecoxib, a selective COX-2 inhibitor, has recently been found to prevent or have therapeutic effects on colon carcinomas, mesothelioma, as well as lung, oral, gastric, and prostate tumors (14, 15). Furthermore, celecoxib also

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Key Words: Apoptosis, celecoxib, cytotoxicity, neuroblastoma, valproic acid.

suppresses the proliferation of neuroblastoma cells and inhibits tumor growth of neuroblastomas (16, 17). Although celecoxib is a COX-2 inhibitor, the mechanism of its antineoplastic effect could be COX-2-dependent or -independent (14, 18). Celecoxib has various functions such as induction of apoptosis and cell cycle arrest, inhibition of anti-apoptotic proteins Mcl-1 and survivin, down-regulation of δNp73, and suppression of E2F1 along with activation of the p73 promoter, which might be related to its anticancer effects (14, 15, 19).

Both VPA and celecoxib are promising for cancer therapy since each has antitumor effects, can be administered orally and can be used for extended periods (2, 20). It is well known that the combination of low doses of cancer chemotherapeutic agents with different modes of action may produce synergistic effects on efficacy and minimize possible side-effects associated with high-dose administration (21). Further, there are reports revealing that VPA could augment the antitumor effects of other chemotherapeutic agents such as 5-aza-2'-deoxycytidine, VP-16, ellipticine, doxorubicin, and cisplatin (2). In addition, celecoxib has also been found to enhance the cytotoxic effect of other chemotherapeutic drugs used in neuroblastoma treatment such as irinotecan, doxorubicin and etoposide (16, 22). However, there is no report investigating the effects of combined VPA and celecoxib on cancer cells. Therefore, in this study, we investigated the effects of combined VPA and celecoxib on neuroblastoma cells, including cell survival, apoptosis, cell cycle, and apoptosis- and cell cycle-related proteins.

Materials and Methods

Cell culture. The human SH-SY5Y neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in minimal essential medium and F12 with 10% fetal calf serum at 37˚C in a 5% CO_2 incubator.

Valproic acid and celecoxib. VPA was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and celecoxib was kindly provided by Pfizer Limited (New York, NY, USA). The drugs were dissolved in dimethyl sulfoxide (Sigma-Aldrich) for the experiments.

Cytotoxic effects of valproic acid or celecoxib on neuroblastoma cells. The sensitivity of neuroblastoma cells to VPA or celecoxib was determined *in vitro* by an MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay. For this assay, 5×10^3 cells were seeded in triplicate wells of a flat-bottomed 24-well microtiter plate and cultured overnight prior to treatment. Cells were exposed to various concentrations of VPA (0-100 mM) or celecoxib (0-100 μM) for 24 or 48 hours. After removal of the drug, cells were incubated for a total of five days. The extent of cell proliferation and viability was determined by the MTT assay. The lethal concentrations (LC) LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50} of VPA and celecoxib (the concentrations at which 90%, 80%, 70%, 60% and 50% of the neuroblastoma cells were killed, respectively) were determined and used for comparison with various combined-treatment conditions as described below.

Cytotoxic effects of combined valproic acid and celecoxib on neuroblastoma cells. To investigate the effects of combined VPA and celecoxib treatment on neuroblastoma cells, various concentrations of VPA (0-100 mM) and celecoxib (0-5 μM) were delivered concomitantly for 24 or 48 hours. Cell proliferation and viability were then determined using the MTT assay described above. The LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50}, as well as the dose enhancement ratio, were determined for various treatment conditions. The dose enhancement ratio was calculated by dividing the VPA dose required to kill the cells during combined VPA and celecoxib treatment by the VPA dose required to kill the cells during VPA treatment alone.

Analysis of apoptosis of neuroblastoma cells induced by valproic acid, celecoxib, and combined valproic acid and celecoxib. The apoptotic fraction of the neuroblastoma cells subsequent to VPA (0, 1, 5 mM), celecoxib (0, 1, 5 μM), or combined VPA and celecoxib treatment for 24 or 48 hours was quantified by flow-activated cell sorter (FACS) flow cytometry using the Annexin V detection Kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All procedures were conducted according to the manufacturer's instructions. Analysis was performed with FACScan flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry System, San Jose, CA, USA).

Analysis of the cell cycle distribution of neuroblastoma cells treated with valproic acid, celecoxib, or combined valproic acid and celecoxib. The cell-cycle distribution of the neuroblastoma cells subsequent to VPA (5 mM), celecoxib (5 μM), or combined VPA and celecoxib treatment for 24 hours was studied by FACS flow cytometry. Briefly, following drug treatment, 1×10^6 cells were trypsinized and washed twice with phosphate-buffered saline (PBS). Cells were stored in 1 mL 80% ethanol/PBS at –20˚C for subsequent experiments. For FACScan analysis, cells were centrifuged at 14,000 rpm for five minutes and washed twice with PBS. Then, cells were incubated with 0.5 mL 0.5% Triton X-100/PBS and 5 μg RNase A for 30 minutes, then stained with 0.5 mL 50 μg/mL propidium iodide/PBS in the dark. Analysis was performed with FACScan flow cytometry.

Whole-cell extract preparation and Western blot analysis. Neuroblastoma cells were treated with VPA (5 mM), celecoxib (5 μM), or combined VPA and celecoxib for 4 hours, and the expression of various proteins was analysed by Western blot analysis. Cells were lysed in a buffer containing 20 mM HEPES (pH 7.6), 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 0.5 μg/ml leupeptin, 1 μg/mL aprotinin, and 100 μg/mL 4-(2-aminoethyl) benzenesulfonyl fluoride. The cell lysate was rotated at 4˚C for 30 minutes, and then centrifuged at 10,000 rpm for 10 minutes; precipitates were discarded. The concentration of protein in the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin used as a reference standard. For western blot analysis, cellular protein (20-50 μg) was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Protein bands were then transferred electrophoretically to PVDF membranes (Micron Separations Inc., Westborough, MA, USA). Membranes were probed with anti-Cox-2, anti-p53, anti-14-3-3σ, anti-p21, anti-p27, anti-Bcl-2, anti-Bax, or anti-β-actin (Santa Cruz Biotechnology), followed by a horseradish peroxidase-conjugated

secondary antibody (Santa Cruz Biotechnology). Detection of antibody reactions was performed with Western blotting reagent ECL (Santa Cruz Biotechnology), with resultant chemiluminescence detected through exposure of the filter to X-ray film. Differences in expression of proteins were analyzed.

Statistical analyses. One-way analysis of variance (ANOVA) by Scheffe's multiple comparison was used for statistical analyses of the extent of cytotoxicity, apoptosis and cell cycle distribution of the neuroblastoma cells induced by various kinds of regimens. Statistical significance was accepted as $p<0.05$.

Results

Valproic acid-induced cytotoxicity of neuroblastoma cells. To investigate the effects of VPA on neuroblastoma cells, they were exposed to various concentrations of VPA (0-100 mM) for 24 or 48 hours. The survival curves for cells treated with VPA are presented in Figure 1A. The effect of VPA was both concentration- and time-dependent, with higher concentrations and longer treatments having significantly greater cytotoxic effects ($p<0.05$). The LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50} were 66.0, 38.2, 23.3, 16.1 and 9.2 mM, respectively, for 24-h VPA treatment, and 45.8, 24.7, 18.3, 12.0 and 5.7 mM, respectively, for 48-h VPA treatment.

Celecoxib-induced cytotoxicity of neuroblastoma cells. To investigate the effects of celecoxib on neuroblastoma cells, they were exposed to various concentrations of celecoxib (0-100 μM) for 24 or 48 hours. The survival curves for cells treated with celecoxib are presented in Figure 1B. The effect of celecoxib was both concentration- and time-dependent, with higher concentrations and longer treatments having significantly greater cytotoxic effects ($p<0.05$). The LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50} were 95.4, 82.6, 65.0, 43.8 and 27.9 μM, respectively, for 24-h celecoxib treatment, and 77.5, 58.1, 43.2, 31.3 and 21.0 μM, respectively, for 48-h celecoxib treatment.

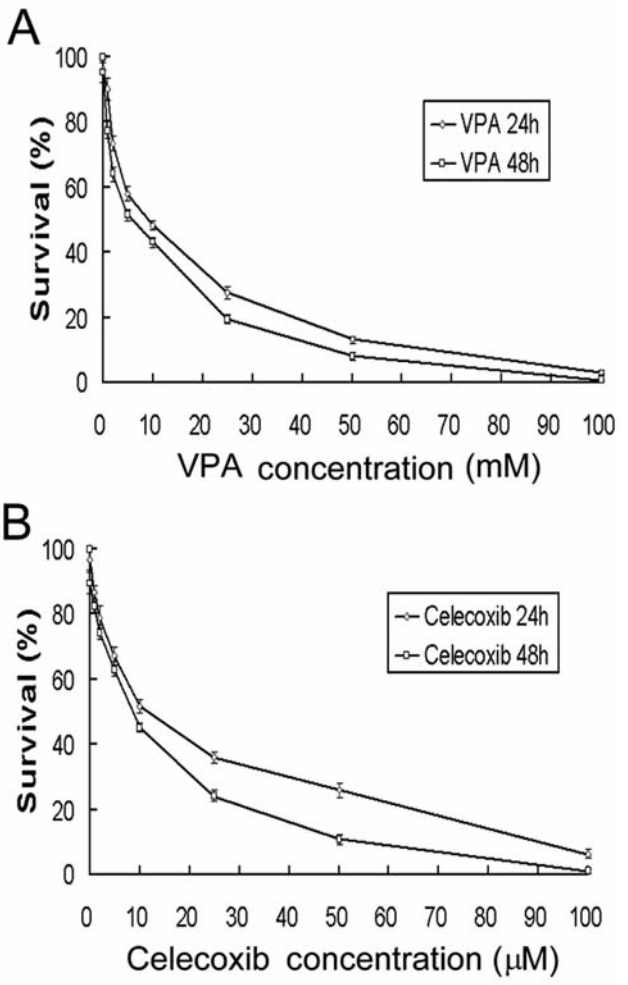

Figure 1. *Cytotoxicity effects of VPA or celecoxib on neuroblastoma cells. Neuroblastoma cells were treated with various concentrations of VPA (0-100 mM) (A) or celecoxib (0-100 μM) (B) for 24 or 48 h. Each point is the average of three independent trials (nine determinations for each concentration) and presented as the mean±standard deviation.*

Combined valproic acid and celecoxib treatment exerted synergistic cytotoxic effects on neuroblastoma cells. To investigate the effects of combined VPA and celecoxib on neuroblastoma cells, they were exposed to various concentrations of VPA and celecoxib concomitantly for 24 or 48 hours. The survival curves after various treatments are shown in Figure 2. Combination treatment induced more cell death than treatment with either VPA or celecoxib, with higher drug dosages and longer exposure times being significantly more effective ($p<0.05$). Tables I and II present the dose enhancement ratios of combined VPA and celecoxib treatment for 24 or 48 hours. For LC_{10}-LC_{50}, the combination treatment of various concentrations of VPA and 5 μM celecoxib for 24 hours had dose enhancement ratios of 2.6-3.4 and that for 48 hours had dose enhancement ratios of 2.7-5.0. The dose enhancement ratios were increased when the dosage of VPA and/ or celecoxib was increased and the drug exposure time was longer.

Combined valproic acid and celecoxib treatment induced more apoptosis than either valproic acid or celecoxib treatment in neuroblastoma cells. The apoptotic fraction of the neuroblastoma cells treated with VPA, celecoxib, or combined VPA and celecoxib for 24 hours was investigated (Figure 3). Apoptosis was concentration-dependent in neuroblastoma cells treated with either VPA or celecoxib ($p=0.0001$). In addition, combination treatment induced more cellular apoptosis than treatment with either VPA or celecoxib, with higher drug dosages and longer exposure times being significantly more effective ($p=0.0001$).

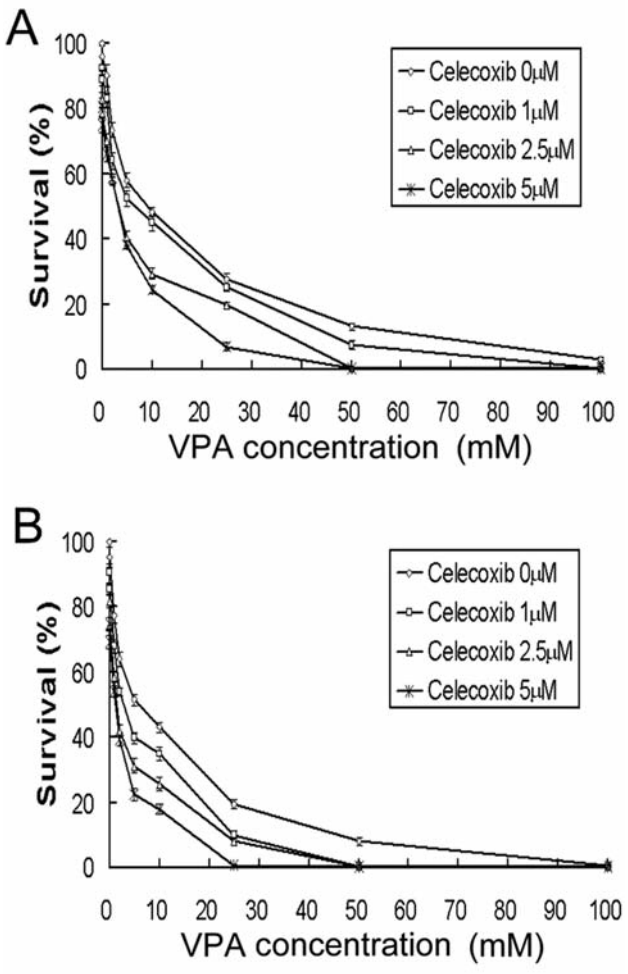

Figure 2. *Cytotoxic effects of combined VPA and celecoxib treatment on neuroblastoma cells. Neuroblastoma cells were treated with various concentrations of VPA (0-100 mM) and celecoxib (0-5 μM) for 24 (A) or 48 (B) h. Each point is the average of three independent trials (nine determinations for each concentration) and presented as the mean±standard deviation.*

Combined valproic acid and celecoxib induced more neuroblastoma cells to accumulate in the G_0/G_1 phase than either valproic acid or celecoxib treatment. The cell-cycle distribution of the neuroblastoma cells subsequent to VPA, celecoxib, and combined VPA and celecoxib treatment for 24 hours was studied (Figure 4). Treatment with 5 mM VPA, 5 μM celecoxib, or combined 5 mM VPA and 5 μM celecoxib for 24 hours increased the fraction of cells in the G_0/G_1 phase and decreased the fraction in the S phase and G_2/M phase, as compared to the control (p=0.0001); there was no difference in the fraction of cells in the S phase between the control and celecoxib treatment (p=0.05). In addition, combined VPA and celecoxib treatment significantly increased the fraction of cells in the G_0/G_1 phase and reduced the fraction in the S phase and G_2/M phase, as compared to VPA or celecoxib treatment alone (p<0.04).

Combined valproic acid and celecoxib increased the expression of p21 and p27 in neuroblastoma cells. Neuroblastoma cells were treated with VPA, celecoxib, and combined VPA and celecoxib for 4 hours, and the expression of various proteins was analysed by Western blot analysis (Figure 5). VPA increased the expression of COX-2 by 2.4-fold as compared to the control; however, celecoxib, and combined VPA and celecoxib suppressed it to 0.59- and 0.51-fold, respectively. VPA, celecoxib, or combined VPA and celecoxib slightly decreased expression of p53 and 14-3-3σ (0.9- and 0.93-fold, respectively). Furthermore, VPA increased the expression of p21 and p27 by 1.8- and 1.7-fold; celecoxib increased their expression by 2.5- and 2.2-fold, respectively; and combined VPA and celecoxib treatment increased p21 and p27 expression by 2.8- and 3.0-fold, respectively. VPA, celecoxib, and combined VPA and celecoxib did not change the expression of Bcl-2. However, VPA increased the expression of Bax by 2.3-fold of the control, celecoxib increased it by 2.9-fold and combined VPA and celecoxib increased it by 2.2-fold.

Discussion

In this study, we found that both VPA and celecoxib induced cytotoxicity and apoptosis in neuroblastoma cells. Such effects were concentration- and time-dependent, and at the LC_{50} was at the millimolar level for VPA and the micromolar level for celecoxib. It is well recognized that combination therapy is necessary to limit the toxicity associated with high doses of a single treatment regimen (21). In the literature, VPA, and celecoxib have been found to enhance the cytotoxic effect of other chemotherapeutic drugs (2, 16); however, there is no study on the combination therapy of VPA and celecoxib. Thus we tested the effect of combined VPA and celecoxib treatment on neuroblastoma cells and found such combination had synergistic effects on the induction of cytotoxicity and apoptosis. The dose enhancement ratio was also dose- and time-dependent, with higher ratios for higher concentrations of drugs and longer drug exposure.

VPA is a kind of HDAC inhibitor and recently HDAC inhibitors have been found to modify the expression of COX-2 in various types of cells (23-25). Inhibition of HDAC by small interfering ribonucleic acid promotes *COX2* gene expression while HDAC overexpression suppresses it (23, 26, 27). Thus the interaction between VPA and celecoxib (a COX-2 inhibitor) might influence their combined cell-killing effect; although the antitumor effects of COX-2 inhibitors

Table I. *Dose enhancement ratios[a] of the LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50} in neuroblastoma cells treated with combined VPA and celecoxib for 24 hours.*

Treatment	LC10[b]	LC_{20}	LC_{30}	LC_{40}	LC_{50}
0 μM Celecoxib + VPA 24 h	66.0 mM	38.2 mM	23.3 mM	16.1 mM	9.2 mM
1 μM Celecoxib + VPA 24 h	46.5 mM	32.2 mM	21.3 mM	13.8 mM	6.7 mM
Enhancement ratio	1.4	1.2	1.1	1.2	1.4
2.5 μM Celecoxib + VPA 24 h	37.8 mM	25.0 mM	9.7 mM	5.1 mM	3.6 mM
Enhancement ratio	1.7	1.5	2.4	3.2	2.6
5 μM Celecoxib + VPA 24 h	22.4 mM	13.9 mM	8.0 mM	4.8 mM	3.6 mM
Enhancement ratio	2.9	2.7	2.9	3.4	2.6

[a]The dose enhancement ratio was calculated by dividing the VPA dose required to kill the cells during combined VPA and celecoxib treatment by the VPA dose required to kill the cells during VPA treatment alone. [b]LC_{10}, LC_{20}, LC_{30}, LC_{40}, LC_{50}: the concentration of VPA at which 90, 80, 70, 60 or 50% of the cells were killed.

Table II. *Dose enhancement ratios[a] of the LC_{10}, LC_{20}, LC_{30}, LC_{40}, and LC_{50} in neuroblastoma cells treated with combined VPA and celecoxib for 48 hours.*

Treatment	LC_{10}[b]	LC_{20}	LC_{30}	LC_{40}	LC_{50}
0 μM Celecoxib + VPA	45.8 mM	24.7 mM	18.3 mM	12.0 mM	5.9 mM
1 μM Celecoxib + VPA 48 h	25.0 mM	19.1 mM	13.1 mM	5.0 mM	3.2 mM
Enhancement ratio	1.8	1.3	1.4	2.4	1.8
2.5 μM Celecoxib + VPA 48 h	23.4 mM	14.9 mM	6.1 mM	3.0 mM	1.8 mM
Enhancement ratio	2.0	1.7	3.0	4.0	3.3
5 μM Celecoxib + VPA 48 h	16.9 mM	7.5 mM	3.9 mM	2.4 mM	1.4 mM
Enhancement ratio	2.7	3.3	4.7	5.0	4.2

[a]The dose enhancement ratio was calculated by dividing the VPA dose required to kill the cells during combined VPA and celecoxib treatment by the VPA dose required to kill the cells during VPA treatment alone. [b]LC_{10}, LC_{20}, LC_{30}, LC_{40}, LC_{50}: the concentration of VPA at which 90, 80, 70, 60 or 50% of the cells were killed.

might be COX-2-dependent or independent (15). We found that VPA increased COX-2 expression significantly in neuroblastoma cells. The regulation of COX-2 expression by VPA might be through the modification of the status of histone acetylation/deacetylation of the *COX2* gene, because the *COX2* promoter is regulated by cytosine methylation and histone deacetylation (28). Whenever the histone of the methylated *COX2* promoter is in the deacetylated state, this gene is inhibited; VPA may increase the histone acetylation of the *COX2* promoter, which therefore increases the promoter activity and *COX2* expression (23, 28). Although VPA enhanced COX-2 expression, the effect was overcome by celecoxib, resulting in suppressed COX-2 expression in neuroblastoma cells treated with combined VPA and celecoxib. VPA-induced COX-2 expression might not significantly disturb the cell-killing effects of combined treatment on neuroblastoma cells.

Although the precise mechanisms underlying the synergistic cell-killing effects of combined VPA and celecoxib treatment remain elusive, both drugs have been noted to modulate the cell cycle and then induce apoptotic cell death (11, 21, 29-31). VPA causes G_0/G_1 accumulation of many malignant cells including endometrial cancer, glioma, melanoma, acute myeloblastic leukemia, carcinoid, and prostate cancer cells (11, 12, 31). In addition, VPA has also been noted to induce G_0/G_1 accumulation in neuroblastoma cells (32). On the other hand, celecoxib has been found to cause G_0/G_1 arrest in most studied cancer cells such as cholangiocarcinoma, colon cancer, prostate cancer, glioblastoma, hepatocellular carcinoma, and oral squamous cell carcinoma cells (21, 30), although it has also been noted to induce G_2/M arrest in normal enterocyte and glioma cells (15, 29). In this study, we found that VPA or celecoxib caused neuroblastoma cells to accumulate in the G_0/G_1 phase, which was consistent to most reports. In

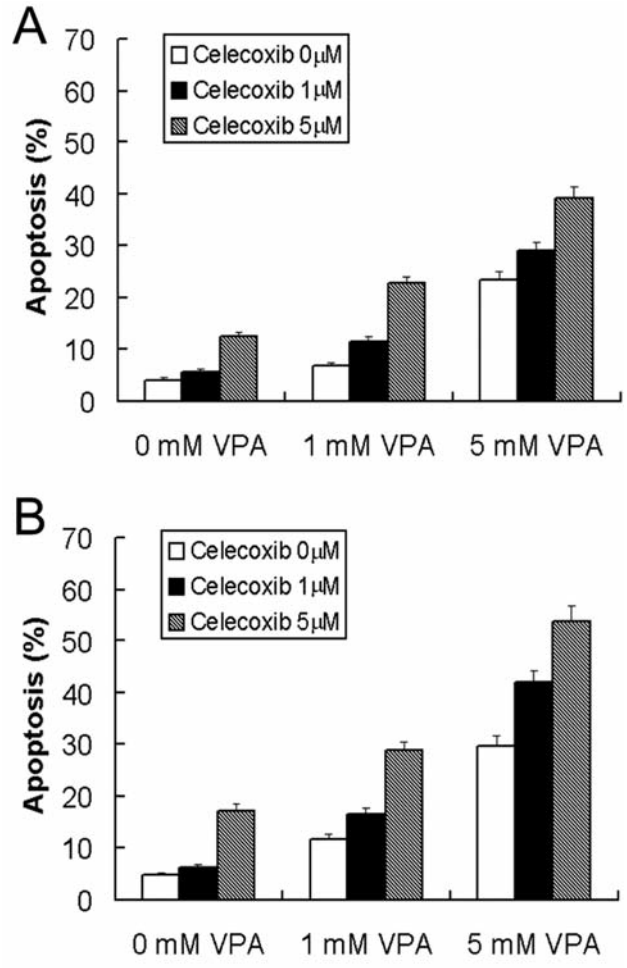

Figure 3. *Apoptosis of neuroblastoma cells subsequent to combined VPA and celecoxib treatment. After treatment with various concentrations of VPA (0-5 mM), celecoxib (0-5 μM), and combined VPA and celecoxib for 24 (A) or 48 (B) h, the apoptotic fraction of neuroblastoma cells was analyzed using a flow-activated cell sorter. Each point is the average of three independent trials (nine determinations for each concentration) and presented as mean±standard deviation. Statistical significance was accepted as p<0.05.*

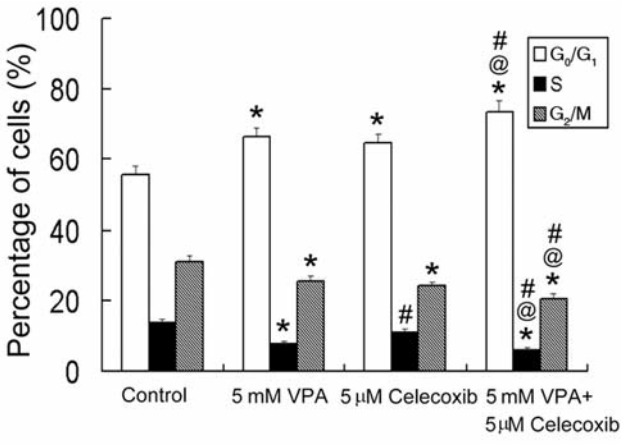

Figure 4. *Cell cycle distribution of the neuroblastoma cells treated with VPA and/ or celecoxib. After treatment with various regimens Control, 5 mM VPA, 5 μM celecoxib, and combined 5 mM VPA and 5 μM celecoxib for 24 h, the cell cycle distribution of neuroblastoma cells was analyzed using a flow-activated cell sorter. Data from three independent experiments are shown as the mean±standard deviation, representing the proportion of cells in the G_0/G_1, S, or G_2/M phases. Statistical significance is accepted as p<0.05: Significantly different from *control, #VPA and @celecoxib treatments.*

addition, combined VPA and celecoxib treatment further increased the fraction of neuroblastoma cells in the G_0/G_1 phase than treatment with either VPA or celecoxib, which indicated that these two agents exerted synergistic effects to cause cell cycle arrest at G_0/G_1 phase.

The cell cycle is a multiple stage process in controlling proliferation involving varieties of regulators, such as retinoblastoma, cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (33). Among these regulators, CDK inhibitors, including p21 and p27, play a key role by negatively regulating the CDK activities during cell cycle transition, with p21 controlling both transition for G_0/G_1 to S phase and transition through G_2/M phase, and p27 controlling the transition from G_0/G_1 to S phase (13, 33). Low expression of p21 or p27 is associated with tumorigenesis, progression, metastasis and poor prognosis in a variety of cancer types (13, 33). Neuroblastomas are often associated with *MYCN* overexpression, which is correlated with tumor growth, invasion and poor prognosis, and, in addition, *MYCN* has been found to suppress the expression of p21 and p27 in neuroblastomas (1, 34, 35). Therefore, any treatment strategy able to increase the expression of *p21* and *p27* might be used to treat neuroblastomas. Previous reports have shown that VPA or celecoxib induces a concomitant increase of *p21* and *p27* gene expression in various tumor cells (12, 13). Our study also found VPA, and celecoxib both activated the expression of p21 and p27 concomitantly in neuroblastoma cells. In addition, we further demonstrated combined VPA and celecoxib treatment induced more p21 and p27 expression than individual drug treatment, which was consistent with there being a higher G_0/G_1 fraction in combination treatment than in single-drug treatment. Both p21 and p27 inhibit a broad range of CDKs, including CDK4/6 and CDK2, and the activities of CDK–cyclin complexes, and thus arrest cell cycle and inhibit proliferation (36, 37). P21 inhibits the kinase activity of CDK4– and CDK6–cyclin D complexes, thus suppressing progression through G_1 phase (37, 38). P21 also inhibits CDK2–cyclin E, with the consequent inhibition of CDK2-dependent phosphorylation of retinoblastoma and the sequestration of E2F1, thus

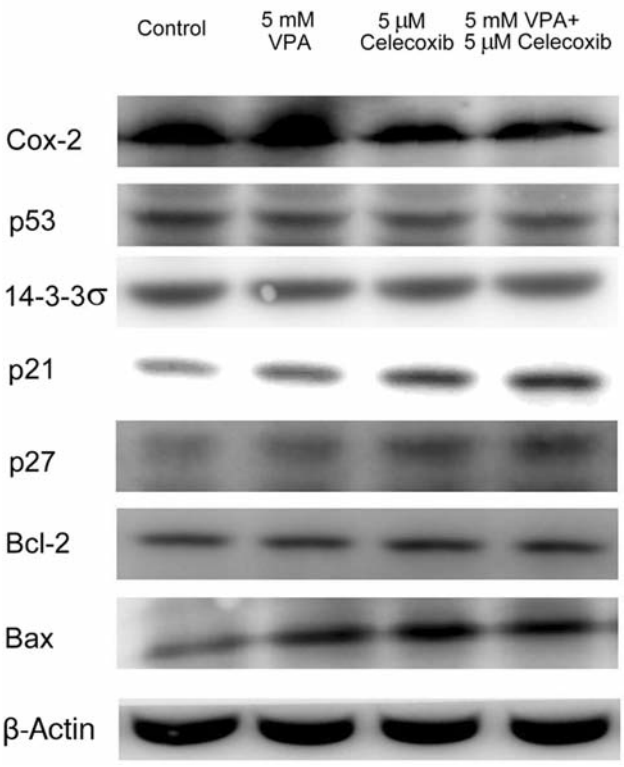

Figure 5. *Expression of various proteins in the neuroblastoma cells treated with valproic acid and/or celecoxib. Western blot analysis of various proteins (COX-2, p53, 14-3-3σ, p21, p27, Bcl-2, Bax, and β-actin (control)) in neuroblastoma cells treated with VPA (5 mM) and/or celecoxib (5 μM) for 4 h.*

inhibiting E2F1-dependent gene transcription and progression into and through the S phase (38). On the other hand, p27 inhibits both cyclin D-CDK4/6 and cyclin E/A-CDK2 to result in the inhibition of G_1/S progression (39). Thus the concomitantly increased p21 and p27 expression might work together to induce cells to accumulate in the G_0/G_1 phase. The increased p21 expression may be regulated *via* p53-dependent or -independent pathways (38). Because p53 and 14-3-3σ expressions were slightly reduced by VPA, celecoxib, and combined VPA and celecoxib treatment, the increased p21 expression likely occurs through the p53-independent pathway.

Induction of apoptosis requires up-regulation of pro-apoptotic activities or down-regulation of anti-apoptotic mechanisms (15). The Bcl-2 family consists of important regulators of cellular apoptosis (15). Bcl-2 can regulate the commitant phase of apoptosis, delay cytochrome *c* release, and thereby inhibit apoptosis (15). In contrast, Bax promotes cellular apoptosis (15). In the literature, VPA, and celecoxib have been found to affect protein expression of the Bcl-2 family; however, their effects are inconsistent (12, 15, 40-42). These two agents may increase, decrease or not change the expression of Bcl-2 and Bax (12, 15, 40-42). In this study, VPA, celecoxib, and combined VPA and celecoxib treatment did not change Bcl-2 expression, but they consistently increased the expression of Bax in neuroblastoma cells, thus the Bcl-2/Bax ratio was shifted to favor apoptosis (15). These data suggested that combined VPA and celecoxib treatment induced apoptosis of neuroblastoma cells through the mitochondria-mediated intrinsic pathway.

In summary, our results showed that combined VPA and celecoxib treatment exerted synergistic effects on the induction of cytotoxicity and apoptosis in neuroblastoma cells. The combination treatment induced more neuroblastoma cells to accumulate in the G_0/G_1 phase than single-agent treatment. Further, cooperation of VPA and celecoxib induced more p21 and p27 expression in neuroblastoma cells than single agent treatment. The increased p21 and p27 expression might be involved in the increased cell accumulation in the G_0/G_1 phase and the increase in cell death when both agents were used together, as compared to either VPA or celecoxib treatment. In addition, the induction of apoptosis by combined VPA and celecoxib treatment was through the mitochondria-mediated intrinsic pathway. The present work suggests the possible therapeutic benefits of combined VPA and celecoxib treatment for neuroblastomas, although more studies are needed before clinical application.

Acknowledgements

This study was supported by research grant FEMH-96-D-015 from Far Eastern Memorial Hospital, Taiwan, R.O.C., awarded to Dr. Yun Chen.

References

1 Park JR, Eggert A and Caron H: Neuroblastoma: biology, prognosis, and treatment. Pediatr Clin North Am *55*: 97-120, 2008.

2 Hrebackova J, Hrabeta J and Eckschlager T: Valproic acid in the complex therapy of malignant tumors. Curr Drug Therapy *11*: 361-379, 2010.

3 Bug G, Ritter M, Wassmann B, Schoch C, Heinzel T, Schwarz K, Romanski A, Kramer OH, Kampfmann M, Hoelzer D, Neubauer A, Ruthardt M and Ottmann OG: Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer *104*: 2717-2725, 2005.

4 Furchert SE, Lanvers-Kaminsky C, Juurgens H, Jung M, Loidl A and Fruhwald MC: Inhibitors of histone deacetylase as potential therapeutic tools for high-risk embryonal tumors of the nervous system of childhood. Int J Cancer *120*: 1787-1794, 2007.

5 Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, O'Brien S, Faderl S, Estrov Z, Giles F, Murgo A, Ladie N, Verstovsek S and Cortes J: Phase 1/2 study of the combination of 5-aza-2'-deoxycytodine with valproic acid in patients with leukemia. Blood *108*: 3271-3279, 2006.

6 Kuendgen A, Schmid M, Schlenk R, Knipp S, Hildebrandt B, Steidl C, Germing U, Haas R, Dohner H and Gattermann N: The histone deacetylase (HDAC) inhibitor valproic acid as monotherapy or in combination with all-*trans* retinoic acid in patients with acute myeloid leukemia. Cancer *106*: 112-119, 2006.

7 Munster P, Marchion D, Bicaku E, Springett G, De Conti R, Chiappori AA, Munster PN, Burton MK, Stromatt S, Allievi C, Angiuli P, Eisenfeld A, Sullivan DM and Daud AI: Phase 1 trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. J Clin Oncol *25*: 1979-1985, 2007.

8 Stockhausen MT, Sjolund J, Manetopoulos C and Axelson H: Effects of the histone deacetylase inhibitor valproic acid on Notch signaling in human neuroblastoma cells. Br J Cancer *92*: 751-759, 2005.

9 Yang Q, Tian Y, Liu S, Zeine R, Chlenski A, Salwen HR, Henkin J and Cohn SL: Thrombospondin-1 peptide ABT-510 combined with valproic acid is an effective antiangiogenesis strategy in neuroblastoma. Cancer Res *67*: 1716-1724, 2007.

10. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, Ning L, Haymart M, Kunnimalaiyaan M and Chen H: Valproic acid activates Notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. Oncologist *12*: 942-951, 2007.

11 Valentini A, Gravina P, Federici G and Bernardini S: Valproic acid induces apoptosis, $p16^{INK4A}$ up-regulation and sensitization to chemotherapy in human melanoma cells. Cancer Biol Ther *6*: 185-191, 2007.

12 Takai N, Desmond JC, Kumagai T, Gui D, Sais JW, Whittaker S, Miyakawa I and Koeffler HP: Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res *10*: 1141-1149, 2004.

13 Liu H, Huang P, Xu X, Liu J and Guo C: Anticancer effect of celecoxib *via* COX-2 dependent and independent mechanisms in human gastric cancer cells. Dig Dis Sci *54*: 1418-1424, 2009.

14 Jendrossek V: Targeting apoptosis pathways by celecoxib in cancer. Cancer Lett 2011.

15 Chen JC, Chen Y, Su YH and Tseng SH: Celecoxib increased expression of 14-3-3σ and induced apoptosis of glioma cells. Anticancer Res *27*: 2547-2554, 2007.

16 Ponthan F, Wickstrom M, Gleissman H, Fuskevåg OM, Segerström L, Sveinbjörnsson B, Redfern CP, Eksborg S, Kogner P and Johnsen JI: Celecoxib prevents neuroblastoma tumor development and potentiates the effect of chemotherapeutic drugs *in vitro* and *in vivo*. Clin Cancer Res *13*: 1036-1044, 2007.

17 Pyrko P, Soriano N, Kardosh A, Liu YT, Uddin J, Petasis NA, Hofman FM, Chen CS, Chen TC, Schönthal AH: Down-regulation of surviving expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2 inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells *in vitro* and *in vivo*. Mol Cancer *5*: 19, 2006.

18 Cui W, Hu SX, Tang ZY and Hu KQ: *In vivo* effects and mechanisms of celecoxib-reduced growth of cyclooxygenase-2 (COX-2)-expressing *versus* COX-2-deleted human HCC xenografts in nude mice. Anticancer Drugs *19*: 891-897, 2008.

19 Lau LM, Wolter JK, Lau JT, Cheng LS, Smith KM, Hansford LM, Zhang L, Baruchel S, Robinson F and Irwin MS: Cyclooxygenase inhibitors differentially modulate p73 isoforms in neuroblastoma. Oncogene *28*: 2024-2033, 2009.

20 Ghsoh N, Vhaki R, Mandal V and Mandal SC: COX-2 as a target for cancer chemotherapy. Pharmacol Rep *62*: 233-244, 2010.

21 Xiao H, Zhang Q, Lin Y, Reddy BS and Yang CS: Combination of atorvastin and celecoxib synergistically induces cell cycle arrest and apoptosis in colon cancer cells. Int J Cancer *122*: 2115-2124, 2008.

22 Kaneko M, Kaneko S and Suzuki K: Prolonged low-dose administration of the cyclooxygenase-2 inhibitor celecoxib enhances the antitumor activity of irinotecan against neuroblastoma xenografts. Cancer Sci *100*: 2193-2201, 2009.

23 Cao D, Bromberg PA and Samet JM: COX2 expression induced by Diesel particles involves chromatin modification and degradation of HDAC1. Am J Respir Cell Mol Biol *37*: 232-239, 2007.

24 Choi YH: Induction of apoptosis by trichostatin A, a histone deacetylase inhibitor, is associated with inhibition of cyclooxygenase-2 activity in human non-small cell lung cancer cells. Int J Oncol *27*: 473-379, 2005.

25 Tong M, Ding Y and Tai HH: Histone deacetylase inhibitors and transforming growth factor- induce 15-hydroxyprostaglandin dehydrogenase expression in human lung adenocarcinoma cells. Biochem Pharmacol *72*: 701-709, 2006.

26 Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, Suzuki H, Hayashizaki Y, Hume DA, Sweet MJ and Ravasi T: LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. FASEB J *20*: 1315-1327, 2006.

27 Yamauchi J, Miyamoto Y, Murabe M, Fujiwara Y, Sanbe A, Fujita Y, Murase S and Tanoue A: Gadd45a, the gene induced by the mood stabilizer valproic acid, regulates neurite outgrowth through JNK and the substrate paxillin in N1E-115 neuroblastoma cells. Exp Cell Res *313*: 1886-1896, 2007.

28 Kikuchi T, Itoh F, Toyata M, Suzuki H, Yamamoto H, Fujita M, Hosokawa M and Imai K: Aberrant methylation and histone deacetylation of cyclooxygenase 2 in gastric cancer. Int J Cancer *97*: 272-277, 2002.

29 Dvory-Sobol H, Cohen-Noyman E, Kazanov D, Figer A, Birkenfeld S, Madar-Shapiro L, Benamouzig R and Arber N: Celecoxib leads to G_2/M arrest by induction of p21 and down-regulation of cyclin B1 expression in a p53-independent manner. Eur J Cancer *42*: 422-426, 2006.

30 Mazzanti R, Platini F, Bottini C, Fantappiè O, Solazzo M and Tessitore L: Down- regulation of the HGF/MET autocrine loop induced by celecoxib and mediated by P-gp in MDR-positive human hepatocellular carcinoma cell line. Biochem Pharmacol *78*: 21-32, 2009.

31 Wedel S, Hudak L, Seibel JM, Juengel E, Tsaur I, Wiesner C, Haferkamp A and Blaheta RA: Inhibitory effects of the HDAC inhibitor valproic acid on prostate cancer growth are enhanced by simultaneous application of the mTOR inhibitor RAD001. Life Sci *88*: 418-424, 2011.

32 Bacon CL, Gallagher HC, Haughey JC, Regan CM: Antiproliferative action of valproate is associated with aberrant expression and nuclear translocation of cyclin D3 during the C6 glioma G_1 phase. J Neurochem *83*: 12-19, 2002.

33 Chang JK, Li CJ, Liao HJ, Wang CK, Wang GJ and Ho ML: Anti-inflammatory drugs suppress proliferation and induce apoptosis through altering expressions of cell cycle regulators and pro-apoptotic factors in cultured human osteoblasts. Toxicology *258*: 148-156, 2009.

34 Eckerle I, Muth D, Batzler J, Henrich KO, Lutz W, Fischer M, Witt O, Schwab M and Westermann F: Regulation of BIRC5 and its isoform BIRC5-2B in neuroblastoma. Cancer Lett *285*: 99-107, 2009.

35 Rounbehler RJ, Li W, Hall MA, Yang C, Fallahi M and Cleveland JL: Targeting ornithine decarboxylase impairs development of *MYCN*-amplified neuroblastoma. Cancer Res *69*: 547-553, 2009.

36 Belletti B, Nicoloso MS, Schiappacassi M, Chimienti E, Berton S, Lovat F, Colombatti A and Baldassarre G: p27 (kip1) functional regulation in human cancer: a potential target for therapeutic designs. Curr Med Chem *12*: 1589-1605, 2005.

37 Liu XP, Tsushimi K, Tsushimi M, Kawauchi S, Oga A, Furuya T and Sasaki K: Expression of p21 (WAF1/CIP1) and p53 proteins in gastric carcinomas: its relationships with cell proliferation activity and prognosis. Cancer Lett *170*: 183-189, 2001.

38 Abbas T and Dutta A: p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer *9*: 400-414, 2009.

39 Kitagawa K, Kotake Y and Kitagawa M: Ubiquitin-mediated control of oncogene and tumor suppressor gene products. Cancer Sci *100*: 1374-1381, 2009.

40 Creason TK, Yuan P, Manji HK and Chen G: Evidence for involvement of ERK, PI3K, and RSK in induction of BCL-2 by valproate. J Mol Neurosci *37*: 123-134, 2008.

41 Sung ES, Kim A, Park JS, Chung J, Kwon MH and Kim YS: Histone deacetylase inhibitors synergistically potentiate death receptor 4-mediated apoptotic cell death of human T-cell acute lymphoblastic leukemia cells. Apoptosis *15*: 1256-1269, 2010.

42 Yagi Y, Fushida S, Harada S, Kinoshita J, Makino I, Oyama K, Tajima H, Fujita H, Takamura H, Ninomiya I, Fujimura T, Ohta T, Yashiro M and Hirakawa K: Effects of valproic acid on the cell cycle and apoptosis through acetylation of histone and tubulin in a scirrhous gastric cancer cell line. J Exp Clin Cancer Res *29*: 149, 2010.

Received April 29, 2011
Revised May 19, 2011
Accepted May 20, 2011