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 G0/G1 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 G0/G1 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/rahabdoid 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 G0/G1 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 isofrom 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
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 6Np73, 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 report investigating the effects of combined VPA and celecoxib in neuroblastoma cells.

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% CO2 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 and 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, the 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) LC10, LC20, LC30, LC40, and LC50 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 LC10, LC20, LC30, LC40, and LC50, 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 MgCl2, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na3VO4, 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.

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 2.7-5.0. 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 (\( p=0.0001 \)).
Combined valproic acid and celecoxib induced more neuroblastoma cells to accumulate in the G₀/G₁ 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 G₀/G₁ phase and reduced the fraction in the S phase and G₂/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 LC₅₀ 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

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.
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 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 G0/G1 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 G0/G1 arrest in neuroblastoma cells (32). On the other hand, celecoxib has been found to cause G0/G1 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 G2/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 G0/G1 phase, which was consistent to most reports. In

### Table I. Dose enhancement ratios of the LC10, LC20, LC30, LC40, and LC50 in neuroblastoma cells treated with combined VPA and celecoxib for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LC10</th>
<th>LC20</th>
<th>LC30</th>
<th>LC40</th>
<th>LC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM Celecoxib + VPA 24 h</td>
<td>66.0 mM</td>
<td>38.2 mM</td>
<td>23.3 mM</td>
<td>16.1 mM</td>
<td>9.2 mM</td>
</tr>
<tr>
<td>1 μM Celecoxib + VPA 24 h</td>
<td>46.5 mM</td>
<td>32.2 mM</td>
<td>21.3 mM</td>
<td>13.8 mM</td>
<td>6.7 mM</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>2.5 μM Celecoxib + VPA 24 h</td>
<td>37.8 mM</td>
<td>25.0 mM</td>
<td>9.7 mM</td>
<td>5.1 mM</td>
<td>3.6 mM</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>1.7</td>
<td>1.5</td>
<td>2.4</td>
<td>3.2</td>
<td>2.6</td>
</tr>
<tr>
<td>5 μM Celecoxib + VPA 24 h</td>
<td>22.4 mM</td>
<td>13.9 mM</td>
<td>8.0 mM</td>
<td>4.8 mM</td>
<td>3.6 mM</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>2.9</td>
<td>2.7</td>
<td>2.9</td>
<td>3.4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

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. LC10, LC20, LC30, LC40, LC50: the concentration of VPA at which 90, 80, 70, 60 or 50% of the cells were killed.

### Table II. Dose enhancement ratios of the LC10, LC20, LC30, LC40, and LC50 in neuroblastoma cells treated with combined VPA and celecoxib for 48 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LC10</th>
<th>LC20</th>
<th>LC30</th>
<th>LC40</th>
<th>LC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM Celecoxib + VPA</td>
<td>45.8 mM</td>
<td>24.7 mM</td>
<td>18.3 mM</td>
<td>12.0 mM</td>
<td>5.9 mM</td>
</tr>
<tr>
<td>1 μM Celecoxib + VPA 48 h</td>
<td>25.0 mM</td>
<td>19.1 mM</td>
<td>13.1 mM</td>
<td>5.0 mM</td>
<td>3.2 mM</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>1.8</td>
<td>1.3</td>
<td>1.4</td>
<td>2.4</td>
<td>1.8</td>
</tr>
<tr>
<td>2.5 μM Celecoxib + VPA 48 h</td>
<td>23.4 mM</td>
<td>14.9 mM</td>
<td>6.1 mM</td>
<td>3.0 mM</td>
<td>1.8 mM</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>2.0</td>
<td>1.7</td>
<td>3.0</td>
<td>4.0</td>
<td>3.3</td>
</tr>
<tr>
<td>5 μM Celecoxib + VPA 48 h</td>
<td>16.9 mM</td>
<td>7.5 mM</td>
<td>3.9 mM</td>
<td>2.4 mM</td>
<td>1.4 mM</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>2.7</td>
<td>3.3</td>
<td>4.7</td>
<td>5.0</td>
<td>4.2</td>
</tr>
</tbody>
</table>

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. LC10, LC20, LC30, LC40, LC50: the concentration of VPA at which 90, 80, 70, 60 or 50% of the cells were killed.
addition, combined VPA and celecoxib treatment further increased the fraction of neuroblastoma cells in the G0/G1 phase than treatment with either VPA or celecoxib, which indicated that these two agents exerted synergistic effects to cause cell cycle arrest at G0/G1 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 G0/G1 to S phase and transition through G2/M phase, and p27 controlling the transition from G2/M to G1 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 G0/G1 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 G1 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
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/CDK2 to result in the inhibition of G1/S progression (39). Thus the concomitantly increased p21 and p27 expression might work together to induce cells to accumulate in the G0/G1 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 commitment 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 G0/G1 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 G0/G1 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.

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References


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![Figure 5. Expression of various proteins in the neuroblastoma cells treated with valproic acid and 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 celecoxib (5 μM) for 4 h.](image-url)


