Vitamin K3 Analogs Induce Selective Tumor Cytotoxicity in Neuroblastoma

Toru Kitano, Hiroyuki Yoda, Keichi Tabata, Motofumi Miura, Masaharu Toriyama, Shigeyasu Motohashi, and Takashi Suzuki

School of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba 274–8555, Japan; Department of Pharmacy, Nihon University Itabashi Hospital; and School of Medicine, Nihon University; 30–1 Oyaguchikami-cho, Itabashi-ku, Tokyo 173–0032, Japan.

Received November 4, 2011; accepted December 23, 2011; published online January 23, 2012

We investigated the cytotoxicity of eight vitamin K3 (VK3) analogs against neuroblastoma cell lines (IMR-32, LA-N-1, NB-39, and SK-N-SH) and normal cell lines (human umbilical vein endothelial cells (HUVEC) and human dermal fibroblasts (HDF)) using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. 2-[2-(2-Hydroxy)ethylthio]-3-methyl-1,4-naphthoquinone (VK3-OCH₃) showed especially potent cytotoxic activities against neuroblastoma cells compared with normal cells. In a Hoechst 33342 staining experiment, apoptotic morphologies characterized by cell shrinkage, nuclear condensation, and nuclear fragmentation were observed in IMR-32 and LA-N-1 cells after 48 h of treatment with 10⁻⁵ M of VK3-OCH₃. To clarify the molecular mechanisms of apoptosis induced by VK3-OCH₃, we examined the expression of apoptosis-related proteins using a Proteome Profiler Array and western blotting. Heme oxygenase (HO)-1 was remarkably increased by VK3-OCH₃ compared with the control (173% in IMR-32 and 170% in LA-N-1 at 24 h). Moreover, caveolin-1 was induced by VK3-OCH₃ at 48 h. In addition, VK3-OCH₃ arrested the cell cycle at the G2/M phase in IMR-32 cells. These results suggest that VK3-OCH₃ exhibited a selective antitumor activity via HO-1-related mechanisms.

Key words vitamin K analog; neuroblastoma; cell cycle; apoptosis; heme oxygenase-1

Neuroblastoma, a tumor originating from neural crest cells, is the most frequent solid neoplasm in children. It is responsible for approximately 15% of all pediatric tumors and is associated with a poor prognosis in children older than 1 year of age. Despite advanced multi-modal therapies, including radiotherapy, surgery, and a variety of chemotherapy regimens, stage 4 neuroblastoma patients have an extremely poor prognosis. About 45% of all patients are diagnosed with advanced stage 4. The 5-year survival rate of patients with neuroblastoma cells. The 5-year survival rate of patients with stage 4 is a poor prognosis. The 5-year survival rate of patients with advanced stage 4. The 5-year survival rate of patients with neuroblastoma is the most frequent solid neoplasm in children. It is responsible for approximately 15% of all pediatric tumors and is associated with a poor prognosis in children older than 1 year of age. Despite advanced multi-modal therapies, including radiotherapy, surgery, and a variety of chemotherapy regimens, stage 4 neuroblastoma patients have an extremely poor prognosis. About 45% of all patients are diagnosed with advanced stage 4. The 5-year survival rate of patients with stage 4 is as low as 20–25%. Therefore, novel therapeutic strategies are urgently needed to improve the overall survival of these patients.

Vitamin K, an essential nutrient, has an important role in normal blood coagulation systems. It acts as a cofactor for plasma proteins such as prothrombin and factors VII, IX, X, and X. In recent years, several investigations have shown that vitamin K also possesses antitumor activity. Chemically synthesized vitamin K3 (2-methyl-1,4-naphthoquinone; VK3) has a potent antitumor effect against various types of carcinoma, including hepatic, oral, pharyngeal, mammary, breast, bladder, and blood cancers in vitro. VK3 induces cell cycle arrest and cell death by inhibiting cde25 phosphatase. 2-[2-(3,3-Dihydroxy)propylthio]-3-methyl-1,4-naphthoquinone (VK3-COOH) reportedly induces the differentiation of neuroblastoma cells. 2-[2-(Hydroxyethylthio)-3-methyl-1,4-naphthoquinone (VK3-OH) inhibits the proliferation of human renal cancer cell by inhibiting cde25 phosphatase and inducing apoptosis. In addition, VK3-OH induced the up-regulation of BAX and down-regulation of B-cell lymphoma-2 (Bcl-2) and Bcl-xL. However, its growth inhibition in normal cells is relatively high and its clinical use for malignant hepatic tumors may be limited.

In this study, we investigated the antitumor activities of VK1, VK3, and 8 kinds of VK3 analogs in neuroblastoma and normal cells to determine their cytotoxities and tumor selectivities.

MATERIALS AND METHODS

Reagents The reagents used in this experiment were vitamin K1 (VK1 (1)) (Wako, Osaka, Japan) and menadione (VK3 (2)) (Wako, Japan). Thioether analogs of VK3 were synthesized as described previously (Fig. 1): VK3 oxide (3), 2-[2-(2-Hydroxy)ethylthio]-3-methyl-1,4-naphthoquinone (VK3-OH (4)), 2-[2-(2-Methoxy)ethylthio]-3-methyl-1,4-naphthoquinone (VK3-OCH₃ (5)), 2-[2-(2-Methoxyethylthio)ethylthio]-3-methyl-1,4-naphthoquinone (VK3-OCH₃ (5)), 2-[2-(3,3-Di-hydroxy)propylthio]-3-methyl-1,4-naphthoquinone (VK3-Diol (7)), 3-(1,4-Dihydro-3-methyl-1,4-dioxo-2-naphthylthio)propionic acid (VK3-COOH (8)), 3-methyl-2-[2,2-dimethyl-1,3-di-oxolan-4-yl]ethylthio]-1,4-naphthoquinone (VK3-Acetal (9)), 2-[2-(tetrahydro-2H-pyran-2-yl)oxy]ethylthio]-3-methyl-1,4-naphthoquinone (VK3-THP (10)).

Cell Lines and Culture Conditions IMR-32, LA-N-1, SK-N-SH (RIKEN Cell Bank), and NB-39 (kindly provided by Dr. Toshimitsu Suzuki, Fukushima Medical University) were maintained in RPMI-1640 medium (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (Invitrogen). Normal cell lines (human umbilical vein endothelial cells (HUVEC) and human dermal fibroblasts (HDF) (Lonza) were maintained in EGM and FGM-2 (Lonza), respectively. The cells were maintained at 37°C/5% CO₂ in a humid environment.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Assay IMR-32, LA-N-1, NB-39, and SK-N-SH (1×10⁴ cells/well) were spread onto 96-well culture plates with phenol red-free RPMI 1640 medium (containing 10% FBS) and maintained for 24 h. HUVEC and HDF (2×10⁴ cells/well) were spread onto 96-well culture plates with phenol red-free RPMI 1640 medium (containing 10% FBS) and maintained for 24 h.
cells/well) cells as well as neuroblastoma cells were spread on each cell culture medium. Compounds (final concentration, $3 \times 10^{-7}$–$1 \times 10^{-4}$ M) and the vehicle were then applied for 48 h. After the addition of 0.5% MTT solution (1/10 of the volume of the medium in the well), incubation was continued for a further 3 h at 37°C in 5% CO$_2$. A volume of stop solution (0.04 N HCl in isopropanol) equal to that of the culture medium was then added to each well, and the absorbance was measured at 570 nm (peak) and 655 nm (bottom) after thorough pipetting to disperse the generated blue formazan. The survival rate was calculated as the percentage of that of the vehicle control.

**Hoechst 33342 Staining** An apoptotic nuclear morphology was observed by staining with Hoechst 33342. IMR-32 and LA-N-1 cells (1×10$^3$/well) were spread onto 6-well cell culture plates with RPMI 1640 medium and incubated at 37°C in 5% CO$_2$ for 24 h. VK3-OCH$_3$ (final concentration 1×10$^{-6}$–10$^{-5}$ M) was added and the incubation was continued for 48 h. Hoechst 33342 solution (final concentration, 0.001% of medium) was applied to the wells for 15 min. The cells were then observed

![Fig. 1. Chemical Structures of VK Analogs](image)

**Table 1. IC$_{50}$ Values (μM) of VK Analogs against Neuroblastoma Cell Lines (IMR-32, LA-N-1, NB-39, and SK-N-SH) and Normal Cell Lines (HUVEC, HDF) in MTT Assay**

<table>
<thead>
<tr>
<th>VK Anlog</th>
<th>IC$_{50}$ (μM)</th>
<th>Neuroblastoma cell lines</th>
<th>Normal cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IMR-32</td>
<td>LA-N-1</td>
<td>NB-39</td>
</tr>
<tr>
<td>VK1</td>
<td>56.81</td>
<td>28.96</td>
<td>&gt;100</td>
</tr>
<tr>
<td>VK3</td>
<td>3.18</td>
<td>4.71</td>
<td>7.09</td>
</tr>
<tr>
<td>VK3-Oxide</td>
<td>2.21</td>
<td>4.13</td>
<td>16.56</td>
</tr>
<tr>
<td>VK3-OH</td>
<td>2.15</td>
<td>0.97</td>
<td>5.97</td>
</tr>
<tr>
<td>VK3-OCH$_3$</td>
<td>2.43</td>
<td>1.55</td>
<td>10.69</td>
</tr>
<tr>
<td>VK3-MOM</td>
<td>1.98</td>
<td>1.3</td>
<td>10.66</td>
</tr>
<tr>
<td>VK3-Diol</td>
<td>2.32</td>
<td>1.6</td>
<td>9.37</td>
</tr>
<tr>
<td>VK3-COOH</td>
<td>6.21</td>
<td>17.53</td>
<td>&gt;100</td>
</tr>
<tr>
<td>VK3-Acetal</td>
<td>2.14</td>
<td>2.53</td>
<td>22.02</td>
</tr>
<tr>
<td>VK3-THP</td>
<td>2.02</td>
<td>3.76</td>
<td>63.88</td>
</tr>
</tbody>
</table>

VK1, vitamin K1; VK3, vitamin K3 (menadione); VK3 oxide; VK3-OH, 2-[(2-hydroxy)ethylthio]-3-methyl-1,4-naphthoquinone; VK3-OCH$_3$, 2-[(2-methoxy)ethylthio]-3-methyl-1,4-naphthoquinone; VK3-MOM, 2-[(2-methoxymethoxy)ethylthio]-3-methyl-1,4-naphthoquinone; VK3-Diol, 2-[(2,3-dihydroxy)propylthio]-3-methyl-1,4-naphthoquinone; VK3-COOH, 3-[(1,4-dihydro-3-methyl-1,4-dioxo-2-naphthylthio)propanoic acid; VK3-Acetal, 3-methyl-2-[(2,2-dimethyl-1,3-dioxolan-4-yl)methylthio]-1,4-naphthoquinone; VK3-THP, 2-[(2-tetrahydro-2H-pyran-2-yl)oxy]ethylthio]-3-methyl-1,4-naphthoquinone.
using a fluorescence microscope (BH2-RFL-T3, Olympus).

**Proteome Profiler Array** IMR-32 and LA-N-1 cells (2×10^6/dish, 3 dishes/sample) were spread onto 60-mm cell culture dishes (Corning) with RPMI 1640 medium and incubated at 37°C in 5% CO2 for 24h. Then, VK3-OCH3 (final concentration, 1×10^-5M) or the vehicle was added for 24h. All immunodetection steps were performed utilizing a Proteome Profiler Array (R&D Systems) in accordance with the manufacturer’s instructions. Briefly, the cells were collected and lysed in lysis buffer. The array was incubated overnight with the diluted lysates (500μg/250μL) at 4°C on a rocking platform shaker. Biotinylated antibody cocktail and streptavidin–horseradish peroxidase (HRP) (1:2000) were added to each array. The blots were detected using an enhanced chemiluminescence (ECL) system (GE Healthcare, U.S.A.) and imaged using a luminoimage analyzer LAS-1000 (FUJIFILM). The

---

**Fig. 2. Cytotoxicity of VK3-OCH3 against Neuroblastoma and Normal Cells**

Cytotoxicity was determined by MTT assay. VK3-OCH3 (3×10^-7–1×10^-4M) was applied for 48h on neuroblastoma (IMR-32, SK-N-SH, LA-N-1, NB-39) and normal (HUVEC, HDF) cells. Vertical axis indicates cell survival % against vehicle control.

**Fig. 3. Changes in Cell Morphology after VK3-OCH3-Induced Apoptosis**

Morphologic changes characteristic of apoptosis (cell shrinkage, nuclear condensation, and nuclear fragmentation) were revealed using Hoechst 33342 staining in IMR-32 (A) and LA-N-1 (B). Cells were exposed to (1×10^-6–1×10^-5M) of VK3-OCH3 for 48h, and phase-contrast (upper) and fluorescence (lower) images were obtained. The white arrows indicate apoptotic cells. Bar, 20μm.
density of the blots was analyzed using the National Institutes of Health (NIH) Image-J software.

**Western Blot Analysis** IMR-32 cells (2×10⁶/dish) were spread onto 60-mm cell culture dishes (Corning) with RPMI 1640 medium and incubated at 37°C in 5% CO₂ for 24 h. Then, VK3-OCH₃ (final concentration, 1×10⁻⁵ M) was added and incubated for 2, 4, 8, 24, or 48 h. The cells were collected and lysed with lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, protease inhibitor cocktail I (1:200), phosphatase inhibitor cocktail II (1:100), and 1 mM dithiothreitol). The protein concentration was determined using a protein assay rapid kit (Wako). Cell lysates containing 20 μg of total protein were loaded onto 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels with Tris/glycine running buffer and transferred to polyvinylidene difluoride (PVDF) membranes. All the immunodetection steps were performed utilizing the SNAP i.d. protein detection system (Millipore, U.S.A.). Membranes were blocked with blocking buffer (0.5% skim milk in TTBS, i.e., 137 mM NaCl, 0.1% Tween 20 and 20 mM Tris–HCl, (pH 7.6)) and incubated with primary antibodies (anti-HO-1, 1:500; anti-phospho-p38/mitogen-activated protein kinase (MAPK), 1:100; anti-p21, 1:20; anti-Bax, 1:250; anti-clusterin, 1:50; anti-caveolin-1, 1:50; anti-cyclin A, [Image 71x82 to 524x592])

**Fig. 4. Alterations in the Expressions of Apoptosis-Related Proteins (Proteome Profiler Array)**

Multiple apoptosis-related proteins were detected using a Proteome Profiler Array in IMR-32 (A-(1)) and LA-N-1 (A-(2)). VK3-OCH₃ (1×10⁻⁵, 24 h) and DMSO (as a vehicle control) were applied. The white rectangles indicate HO-1 activation. (A) Array images. (B) Alteration rate as a percentage relative to the vehicle control.
April 2012 621

1:50; anti-cyclin B1, 1:50; and anti-β-tubulin, 1:150; diluted in TTBS containing 5% bovine serum albumin (BSA) and 0.1% sodium azide for 10 min and HRP-conjugated secondary antibody (1:3000, diluted in 0.5% skim milk) for 10 min at room temperature. The signal was detected using an ECL western blotting detection system and imaged using a lumino image analyzer (LAS-1000; FUJIFILM, Japan). The density of the bands was analyzed using NIH Image-J software.

**Cell Cycle Analysis** IMR-32 cells (1×10^6 cells/well) were spread onto 6 well cell culture plate with RPMI-1640 medium and incubated at 37°C/5% CO₂ for 24 h. VK3-OCH₃ (final concentration 1×10⁻⁶–1×10⁻⁵ M) or the vehicle were added and the cells were incubated for 48 h. The cells were collected and washed with PBS. Cells were then fixed with ice-cold 70% ethanol for 2 h. After washing with PBS, cells were treated with 0.25 mg/mL RNase solution for 30 min at 37°C. A propidium iodide (PI) solution was added (final concentration 50 μg/mL) and the cells were incubated for 30 min at 4°C in the dark. The cell samples were analyzed with a FC500 flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.) using the FL3 range.

**RESULTS**

**Cytotoxic Analysis between Neuroblastoma Cell Lines and Normal Cell Lines** At first, we examined the cytotoxicities of 10 compounds against neuroblastoma and normal cells using an MTT assay (Table 1). VK3 (2), VK3-OH (4), VK3-OCH₃ (5), VK3-MOM (6) and VK3-Diol (7) showed potent cytotoxicities against all the neuroblastoma cell lines (IMR-32, LA-N-1, NB-39, and SK-N-SH). Among these compounds, VK3 and VK3-OH also exhibited cytotoxicity in HUVEC as well as the neuroblastoma cell lines. On the other hand, VK3-OCH₃ showed little cytotoxicity in normal cells. The IC₅₀ values of VK3-OCH₃ were 1.55–10.69 μM against the neuroblastoma cell lines and 87.11 μM and 26.24 μM against the HDF and HUVEC cell lines, respectively (Table 1, Fig. 2). These data indicate the selective cytotoxicity of VK3-OCH₃ in neuroblastoma cell lines versus normal cell lines. Therefore, we focused on VK3-OCH₃ in all subsequent studies.

**Analysis of Apoptosis Induction in Neuroblastoma Cell Lines Using VK3-OCH₃** To clarify whether VK3-OCH₃ induces apoptosis in neuroblastoma cell lines (IMR-32, LA-N-1), we observed cellular and nuclear morphological changes using Hoechst 33342 staining. As shown in Fig. 3, apoptosis characterized by cell shrinkage, nuclear condensation, and nuclear fragmentation was observed in IMR-32 and LA-N-1 cells treated for 48 h with VK3-OCH₃ in a concentration dependent manner (1×10⁻⁶–1×10⁻⁵ M).

**Proteome Profiler Array Using VK3-OCH₃** We attempted to define the mechanisms of cytotoxicity induced by VK3-OCH₃ in human neuroblastoma cell lines (IMR-32, LA-N-1). Change in apoptosis related proteins were determined using a Proteome Profiler Array. VK-3OCH₃ (1×10⁻⁵ M, 24 h) remarkably induced heme oxygenase (HO)-1 compared with the control (173% in IMR-32 and 170% in LA-N-1) (Fig. 4).

**Western Blot Analysis** Subsequently, we examined the expressions of proteins in signal pathway related to HO-1 using western blot analyses over time. HO-1 expression was remarkably increased at 8–24 h after the application of VK3-

---

**Fig. 5. Western Blotting Analysis of HO-1-Related Proteins**

HO-1 (32kDa), p-p38-MAPK (43kDa), caveolin-1 (22kDa), p21 (21kDa), clusterin (70kDa), Bax (20kDa), cyclin A (54kDa), and cyclin B1 (60kDa) were detected using western blotting using an antibody against each protein in IMR-32 (A) and LA-N-1 (B). VK3-OCH₃ (1×10⁻⁵ M) was applied for 0–48 h. The expression of β-tubulin (55kDa) was used as a loading control.
after treatment with VK3-OCH3 for 48 h using vehicle, B (green): VK3-OCH3 (1 μM), C (blue): VK3-OCH3 (3 μM), D (red): VK3-OCH3 (10 μM). The percentage of each population was shown below.

Cell Cycle Analysis Using Flow Cytometry We also examined the cell cycle status of IMR-32 cells treated with VK3-OCH3 for 48 h using flow cytometry (Fig. 6). The population of G2/M was significantly increased by treatment with 10 μM of VK3-OCH3. Thus, VK3-OCH3 induced G2/M arrest in IMR-32 cells.

DISCUSSION

VK3 analogs have been previously reported to exhibit antitumor activity in human tumor cells, and VK3-OH reportedly inhibited the growth of hepatoma and Hela cells. We investigated whether VK3-analogs might induce antitumor activity without causing toxicity in normal cells. VK3-OH showed a high level of cytotoxicity against four neuroblastoma cell lines (IMR-32, LA-N-1, NB-39, and SK-N-SH), but it was also highly cytotoxic against normal cell lines (HUVEC, HDF). Moreover, VK3-OCH3, VK3-MOM, and VK3-Diol showed high cytotoxic activities against four neuroblastoma cell lines, but these VK3-analogs (especially VK3-OCH3: HUVEC (IC50, 26.24 μM), HDF (IC50, 87.11 μM)) have a lower cytotoxicity against normal cell lines (Table 1, Fig. 2).

Next, we investigated whether these cytotoxicities against neuroblastoma cells were caused by the induction of apoptosis. We clarified that apoptosis, characterized by cell shrinkage, nuclear condensation, and nuclear fragmentation, was induced in two neuroblastoma cell lines (IMR-32 and LA-N-1) after treatment for 48 h with VK3-OCH3 (Fig. 3).

VK3-OCH3 remarkably up-regulated heme oxygenase (HO)-1 (Fig. 4). HO is a microsomal enzyme that catalyzes the first, rate-limiting step in the degradation of heme and plays an important role in the recycling of iron. HO cleaves the α-meso carbon bridge of heme, yielding equimolar quantities of carbon monoxide (CO), Fe2+ ions, and biliverdin. The overexpression of HO-1 has been reported to augment apoptosis in breast cancer and astroglia, and the anti-apoptotic effects of HO-1 have been reported colon cancer, thyroid cancer, and gastric cancer. These contradictory data are related to the effect of HO-1 on apoptosis and suggest that the final output does not depend on HO-1 alone, but is rather determined by a mutual balance of several players.

HO-1 related signal transduction has been previously described. CO induced by HO-1 stimulates p38-MAPK through the induction of soluble guanyl cyclase (sGC) and the elevation of the cGMP level. Our data also suggested that HO-1 was induced and that p38-MAPK tended to be activated despite the different expressions of p21, clusterin, and Bax between IMR-32 and LA-N-1 after the administration of VK3-OCH3. Moreover, caveolin-1 was up-regulated at 48 h after peak level of HO-1 at 24 h (Fig. 5). Reportedly, p38-MAPK activated by HO-1 also enhances the expression of caveolin-1, which decreases cyclin A and consequently induces growth arrest. Our data also showed the G2/M arrest of the cell cycle in IMR-32 cells. However, cyclin A did not decrease despite increase of caveolin-1 and occurring G2/M arrest. To explain the discrepancy and to clarify the molecular mechanisms in detail, further studies are needed.

In conclusion, our present data suggest that VK analogs, particularly VK3-OCH3, induce G2/M arrest and apoptosis through HO-1 related signal transduction in neuroblastoma cells and exhibit less cytotoxicity in normal cell lines. Clarification of the signals involved may lead to the development of new molecular-targeted therapies for neuroblastoma, and VK3 analogs appear to be promising antitumor agents.

Acknowledgements This work was supported by the ‘Academic Frontier’ Project for Private Universities, a matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) 2007–2010, and a joint research grant from the School of Pharmacy Nihon University.

REFERENCES

5) Cranenburg EC, Schurgers LJ, Vermeer C. Vitamin K: the co-


