4'-O-β-d-Glucopyranosyl-4-hydroxy-3,3',5-trimethoxychalcone derived from *Brassica rapa* L. induces cell cycle arrest and apoptosis in neuroblastoma cells.

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Abstract

Introduction: Neuroblastoma is one of the most commonly encountered malignant solid tumors in the pediatric age group. We examined the antitumor effects of chalcone glycosides derived from *Brassica rapa* L against neuroblastoma cell lines.

Materials and Methods: We used the WST-8 assay to evaluate the cytotoxicity of the glycosides against human neuroblastoma cell lines and normal cell lines. We also performed analysis of cell cycle arrest by flow cytometry, and examined the expression levels of cell cycle-and apoptosis-related proteins by western blot analysis.

Results: One of the derivative compounds (compound 6,4'-O-β-d-glucopyranosyl-4-hydroxy-3,3',5-trimethoxychalcone) was found to exert cell cytotoxicity against neuroblastoma cells. Examination of the effects on the cell cycle revealed cell cycle arrest in the G0/G1 phase. Furthermore, exposure of the cells to compound 6 was associated with decreased protein expression levels of cyclin D, phospho-retinoblastoma protein (Rb) and E2 factor (E2F), as evaluatedby western blot analysis. In addition, a Hoechst 33342 staining experiment revealed apoptosis, characterized by cell shrinkage, nuclear chromatin condensation and fragmentation. Annexin V-propidium iodide (PI) double staining also showed an increase in the number of early apoptotic cells. Compound 6 induced activation of executioner caspases (caspase-3 and caspase-7) and Poly (ADP-ribose) polymerase (PARP) cleavage.

Conclusion: Compound 6 exerted inhibition of cell growth of neuroblastoma cells by inducing G0/G1 arrest and caspase-dependent apoptosis. This compound may offer promise for development as a useful drug for the treatment of advanced neuroblastoma.

Keywords: *Brassica rapa L*, Chalcone glycosides, Neuroblastoma, Cell cycle, Apoptosis.

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Introduction

Neuroblastoma is one of most commonly encountered malignant solid tumours in the paediatric age group. It is an embryonal tumor derived from the neural crest, involving the sympathetic nervous system and adrenal medulla [1]. Neuroblastoma accounts for 15% deaths of all childhood cancers. The reported 5-year overall survival rate among patients with stage 4 disease (International Neuroblastoma Staging System (INSS)) [1,2] is as low as 31%, and still has a poor prognosis rate ; thus, this cancer still carries a very poor prognosis [3]. Most advanced neuroblastoma receive multidisciplinary therapy, including intensive chemotherapy, radiotherapy, surgery, and bone marrow transplantation (BMT) [4-6]. However, these intensive therapies can have severe side effects. Therefore, it is necessary to be safer and more effective novel therapeutic agents are needed for the treatment of high risk neuroblastoma patients.

The cell cycle is regulated by cyclin-dependent kinases (CDKs) that are activated by cyclins [7]. The activities of the cyclin-CDK complexes are regulated by cyclin-dependent kinase inhibitors (CDKIs) and multiple signal transduction pathways in the cell cycle [7]. Differentiation of neurons is involved in cell cycle regulation, including in the G1 phase, and it is reported that dysregulation of the G1 checkpoint is an important cell cycle aberration in neuroblastoma [8-10]. Amplification of the G1-phase genes, such as cyclin D1 and CDK4 occurs frequently in neuroblastomas [11,12]. The involvement of cell cycle regulators, such as cyclin D1 and CDK4/6, in neuroblastoma is an important factor driving tumorigenesis [11,13], and inhibition of these regulators may serve as an effective strategy for treating neuroblastomas.

Chalcones are open-chain flavonoids,that are biosynthesized in a variety of plant species [13]. They are known to exert cytotoxic activities through multiple mechanisms, including

cell cycle arrest, angiogenesis inhibition and apoptosis induction [14]. In previous studies, chalcone glycosides derived from plants belonging to *Brassica rapa* L (*Hidabeni* in Japanese) were demonstrated to show inhibitory effect against NO production [15,16], antigen-stimulated degranulation [17] and induce of neurite outgrowth [18]. Chalcone derivatives similar in structure to aglycon in the present study have also been reported to exert antitumor effects in lung cancer cell lines [19]. However, there are few reports describing the in-depth mechanistic basis underlying the cytotoxic and/or anti-proliferative activities exhibited by the chalcone glycosides. In this study, we examined the cytotoxicity of chalcone glycosides derived from *Brassica rapa* L, and investigated the important mechanisms underlying the antitumor effects of 4'-O-β-d-glucopyranosyl-4-hydroxy-3,3',5-trimethoxychalcone (compound 6).

Materials and Methods

The chemical structures of the chalcone glycosides are shown in Figure 1. They were prepared in the laboratories of Dr. Kotetsu and Dr. Nishina. Stock 50-mM solutions of each compound were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA), stored at -20° C, and then diluted as needed for addition to the cell culture medium. Cisplatin was purchased from Wako pure chemical industries (Osaka, Japan).

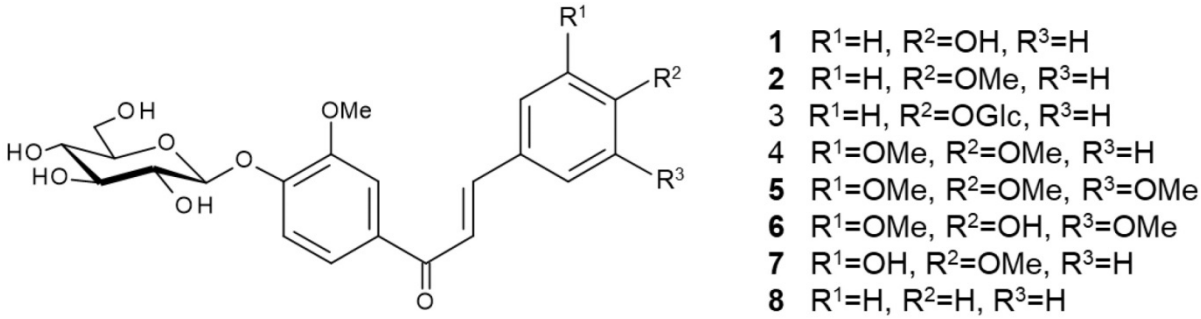

Figure 1. *Chemical structures of the chalcone glycosides derived from Brassica rapa L.*

Cell lines and culture conditions

Human neuroblastoma cell lines (IMR-32, LA-N-1, and SK-N-SH, provided by RIKEN Cell Bank, and NB-39, kindly provided by Dr. Toshimitsu Suzuki, Fukushima Medical University) were maintained in RPMI-1640 medium (Life Technologies Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, UT, USA) at 37° C in a humidified incubator in a 95% air/5% CO_2 atmosphere. Normal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVECs), also obtained from Lonza Japan, were cultured in FGM-2 (Lonza Japan, Tokyo, Japan) and EGM-2 (Lonza Japan), respectively, under the same culture condition as that described above.

Viability assay (WST-8)

Neuroblastoma cell lines (1×10^4 cells/well) were prepared in a 96-well culture plate and incubated for 24 h. The cells were treated with chalcone glycoside derivatives, or DMSO as vehicle control, for 48 h. Then, CCK-8 (Dojindo, Kumamoto, Japan) was added to the wells at 10% of the volume of the medium, and incubation was continued for 3 h at 37°C/5% CO_2. The absorbances were measured at 450 nm. The cell survival rate was calculated as the percentage of viable cells in the treated group versus that in the vehicle control group. The normal control cells (2×10^4 cells/well) were plated on to the respective culture media, with the assay performed in the same way as that described above. The experiments were repeated in triplicate.

Cell cycle analysis (Propidium Iodide Staining)

The cell cycle status was evaluated by quantifying DNA fragment staining with PI (Merck Millipore, Darmstadt, Germany). NB-39 cells (1×10^6 cells/well) were cultured in serum-free medium in the wells of a 6-well culture plate for 24 h. Thereafter, serum was added to the medium and the cells were treated with compound 6 (final concentration: 10 and 30 μM) or vehicle control for 24 and 48 h. Then, the cells were collected and washed twice with ice-cold PBS and fixed in ice-cold 70% ethanol for 2 h. The fixed cells were centrifuged and washed twice with PBS, suspended in 0.25 mg/ml Ribonuclease A (Sigma-Aldrich), and incubated in a shaker at 37° C for 30 min. Then, the cells were stained with PI (final concentration: 50 μg/ml) and incubated in the dark for 30 min. Finally, the cell samples were analyzed with a FC 500 flow cytometer (Beckman Coulter, CA, USA) in the FL3 range. The experiments were repeated in triplicate.

Hoechst 33342 staining

Apoptotic nuclear morphology was observed by staining with Hoechst 33342 (Sigma-Aldrich). NB-39 cells (1×10^5 cells/well) were plated on to the wells of a 6-well culture plate and incubated for 24 h. The cells were treated with compound 6 (final concentration: 1, 10, and 100 μM) or vehicle control for 48 h, followed by addition of Hoechst 33342 solution (final concentration: 0.001% of the medium) to the wells. The wells were allowed to stand for 15 min and then the cells were observed under a fluorescence microscope IX-71 (Olympus, Tokyo, Japan). The experiments were repeated in triplicate.

Annexin V-Propidium iodide double staining analysis (Flow cytometry)

Early apoptosis was detected using the Alexia Fluor® 488 annexin V/Dead Cell apoptosis kit (Life technologies Invitrogen). NB-39 cells (1×10^6 cells/well) were plated on to the wells of a 6-well culture plate, incubated for 24 h, and then treated with compound 6 (final concentration: 1, 10, and 30 μM) or vehicle control for 48 h. Then, the cells were collected and washed with ice-cold PBS and centrifuged after stirring, and the supernatants were discarded. The cell sediments were washed with annexin-binding buffer and stained with annexin V-Alexa Fluor® 488 and PI for 15 min. The cell samples were analyzed with a FC500 flow cytometer (Beckman Coulter) in the FL1 range and FL4 range. The experiments were repeated in triplicate.

Preparation of subcellular fractions and western blotting

Whole-cell proteins were obtained in an extraction buffer. NB-39 cells (2×10^6 cells/dish) were plated on to 60-mm culture dishes, incubated for 24 h, and treated with compound 6 (final concentration: 20μM) for 0-72 h (0 h refers to untreated dishes). The cells were collected and washed with ice-cold Tris-buffer saline (TBS), and lysed in an extraction buffer. They were disrupted by sonication twice for 30 s each, and centrifuged at 20,630 × g for 10 min at 0°C. Protein concentrations in the cell supernatants were determined with a Protein Assay Rapid Kit (Wako), using BSA as reference. The subcellular fractions, as loading samples containing 10 μg of the proteins, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). After blocking with 5% skim milk or BSA for 1 h at room temperature, the membranes were probed with the primary antibodies overnight at 4°C. After another wash, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The blots were examined using an ECL system (GE Healthcare, Buckinghamshire, UK) and imaged using the LUMINESCENT IMAGE ANALYZER LAS-1000 plus (FUJIFILM, Tokyo, Japan). The densities of the bands were analyzed using the NIH Image-J software (U.S. National Institutes of Health, MD, USA,). The primary antibodies used were as follows: anti-caspase-3 (9665), anti-cleaved caspase-3 (6664), anti-caspase-7 (9494), anti-Rb (9313), anti-phospho-Rb (9307), (Cell Signaling Technology, Danvers, MA, USA), anti-PARP (611038), anti-Cyclin D1 (610280), anti-Cyclin E (551159), anti-cdk-4 (559677), (BD Biosciences, San Jose, CA, USA), anti-cdk2 (sc-748), anti-cdk-6 (sc-177), anti-p27 (sc-528), anti-E2F-1 (sc-48334), (Santa Cruz Biotechnology, TX, USA), and anti-β-tubulin (T4026), (Sigma-Aldrich).

Statistical analysis

Data are expressed as means ± SEM (n=3). Significance testing was performed by analysis of variance (ANOVA), followed by Bonferroni's test for comparing three or more data.

Results

Cytotoxicity

We investigated the cytotoxicity of the chalcone glycosides in Figure 1 on four neuroblastoma cell lines and two normal control cell lines by the CCK-8 assay. Compound 6 was found to exert the most potent cytotoxicity against two of the neuroblastoma cell lines, with 50% inhibitory concentration (IC50) values for LA-N-1 and NB-39 of 15.3 and 19.7 μM, respectively in Table 1. Furthermore, compound 6 was also demonstrated to exhibit selective cytotoxicity against neuroblastoma cells as compared to normal control cells.

Table 1. *IC50 values (μM) of the chalcone glycosides derived from Brassica rapa L. for cytotoxicity against neuroblastoma cell lines and normal control cells.*

Compounds		Cytotoxicity IC50 values (μM)					
		Neuroblastoma cell lines			Normal cells		
		IMR-32	LA-N-1	NB-39	SK-N-SH	HUVEC	NHDF
1		>100	72.6	>100	>100	>100	>100
2		71.2	26.1	77.3	84.6	75.5	>100
3		>100	>100	>100	>100	>100	>100
4		>100	34.1	90.1	>100	>100	>100
5		85.5	24 .1	94.5	>100	>100	>100
6		93.5	15.3	19.7	73.2	>100	>100
7		93.4	25.8	78.6	80.5	92.2	>100
8		>100	44.6	>100	>100	99	>100
IC50: 50% inhibitory concentration							

Cell cycle arrest

We examined the cell cycle status of NB-39 cells treated with compound 6 by flow cytometry after PI staining for DNA fragments in Figure 2. Exposure of the cells to 10-30 μM of compound 6 for 48 h induced a significant decrease in the population of cells in the S and G2/M phase, and increase in the population of the cells in the G0/G1 phase in Figure 2B. Thus, a tendency towards increase in the population of cells in the G0/G1 phase was observed in the cells treated with compound 6. We examined the expression levels of the cell cycle- related proteins involved in the cell cycle progression to the G0/G1 phase by SDS-PAGE and western blot analysis in Figure 3. Time-dependent decreases in the expression levels of cyclin D, cyclin E, CDK 2, phospho-Rb and E2F-1, and significant increase in the expression level of only p27 were observed. In particular, the protein levels of cyclin D, phospho-Rb and E2F-1 began to show significant fluctuations after 24 h.

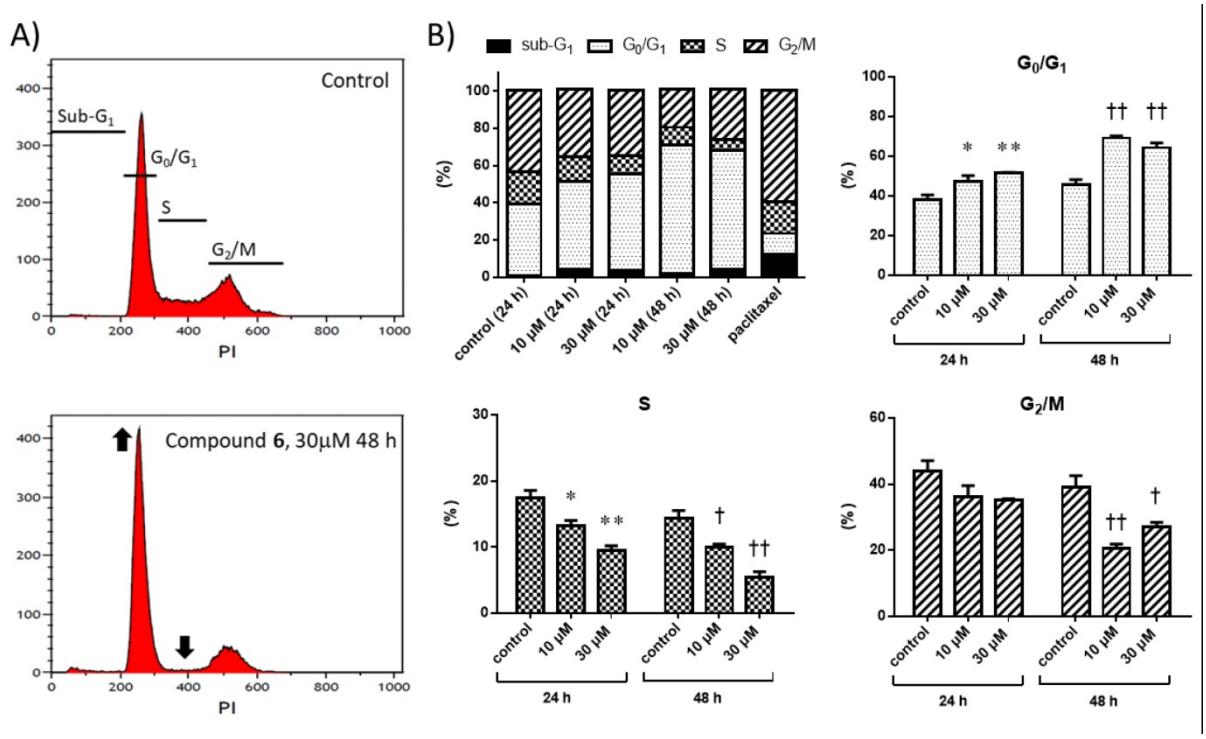

Figure 2. *Analysis of the cell cycle status by flow cytometry. NB-39 cells were treated with compound 6 (10, 30 μM) or DMSO (as a vehicle control) for 24 and 48 h. (A) Histograms of the flow cytometry. (B) Percentage of cells at each stage of the cell cycle, as analyzed in A. *p<0.05, **p<0.01 versus vehicle control (24 h), †p<0.05, ††p<0.01 versus vehicle control (48 h). Paclitaxel was used as the control for induction of G2/M arrest.*

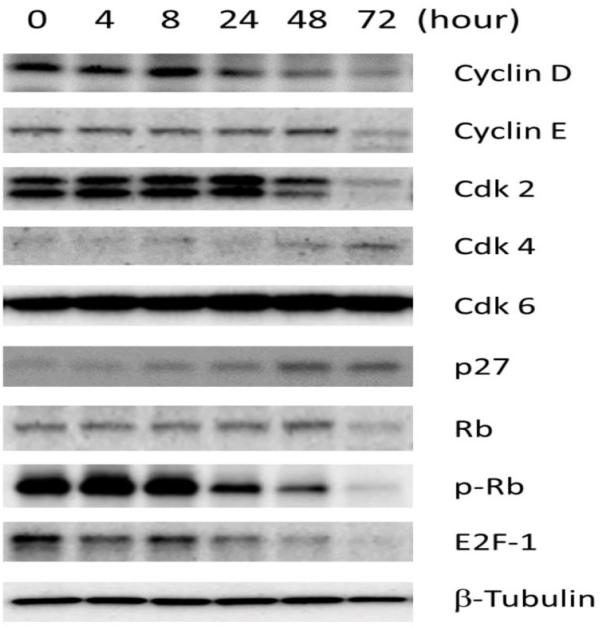

Figure 3. *Western blot analysis for cell cycle-related proteins. NB-39 cells were treated with compound 6 (20 μM) for 0-72 h. The expression levels of the cell cycle-related proteins were assessed by western blotting; β-tubulin was used as the loading control.*

Apoptosis

To detect whether compound 6 induced apoptosis in the neuroblastoma cells, we observed the cellular and nuclear morphological change by Hoechst 33342 staining. Cell shrinkage, nuclear chromatin condensation and nuclear fragmentation, all characteristic features of apoptosis, were observed in the NB-39 cells treated with compound 6 (Figure 4, arrows at 10 and 100 μM): the severity of the apoptotic changes increased in a dose-dependent manner (Figure 4). Subsequently, the NB-39 cells treated with compound 6 were subjected to flow-cytometric analysis after annexin V-PI double staining (Figure 5). An increase in the percentage of early apoptotic cells was observed after treatment with the compound at 30 μM (Figure 5B). We examined the cellular expression levels of apoptosis-related proteins by SDS-PAGE and western blot analysis (Figure 6). Decrease in the expressions of caspase-3 and caspase-7 and increase in cleavage of the same caspases were observed in a time-dependent manner. Subsequently, cleavage of PARP was detected. Furthermore, decrease in the protein expression levels of survivin and XIAP was also observed.

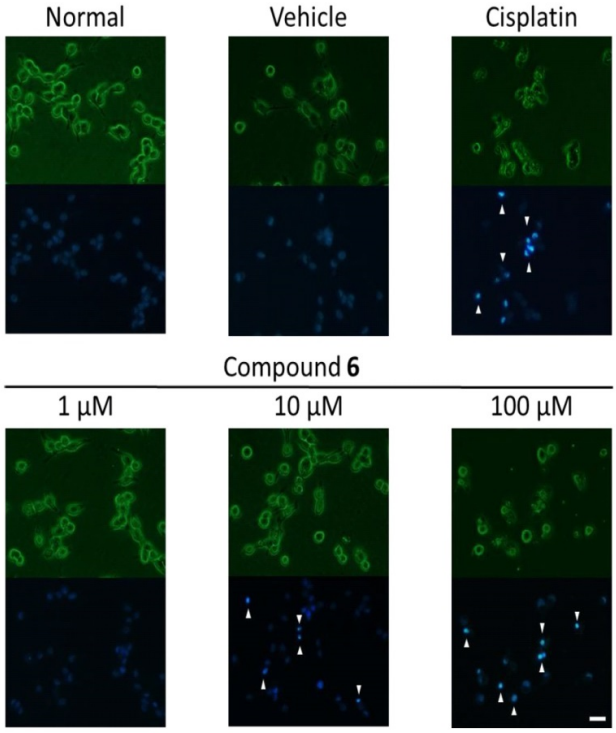

Figure 4. *Morphological observation following Hoechst 33342 staining. NB-39 cells were treated with Compound 6 (1, 10, 100 μM), cisplatin (100 μM) or DMSO (as a vehicle control) for 48 h. Phase-contrast images (upper) and fluorescence images (lower) were obtained. White arrows at 10 and 100 μM indicate the morphological features of apoptosis, including cell shrinkage, nuclear chromatin condensation and nuclear fragmentation. Scale bar: 30 μm.*

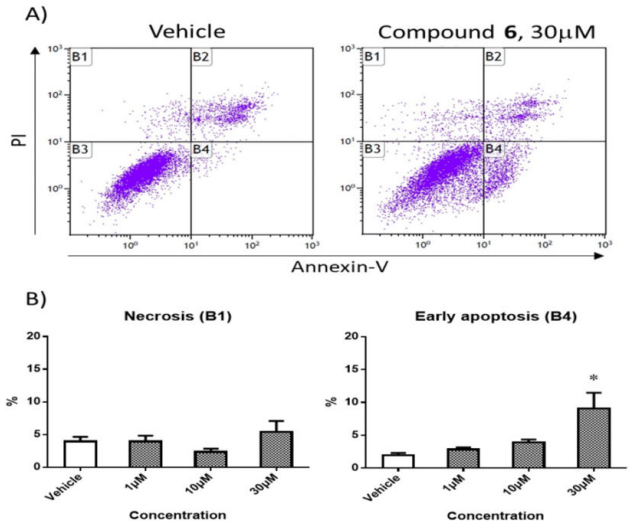

Figure 5. *Analysis of early apoptotic cells by flow cytometry. NB-39 cells were treated with compound 6 (30 μM) or DMSO (as a vehicle control) for 48 h. (A) B1: necrotic cells; B2: late apoptotic and necrotic cells; B3: viable cells; B4: early apoptotic cells. Vertical axis indicates PI-stained cells (FL4 Log) and horizontal axis indicates annexin V-Alexa Fluor® 488-stained cells (FL1 Log). (B) Percentages of the cell populations in each area of A. *p < 0.05, **p < 0.01 versus vehicle control.*

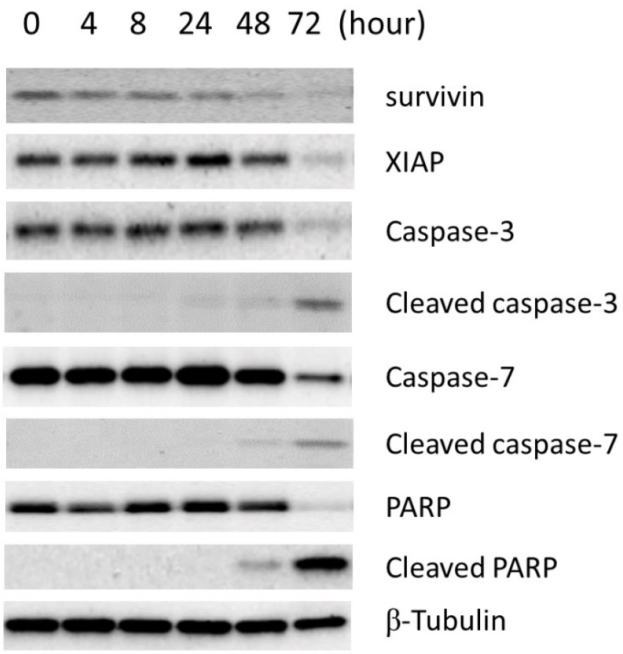

Figure 6. *Western blot analysis for apoptosis-related proteins. NB-39 cells were treated with compound 6 (20 μM) for 0-72 h. The expression levels of the apoptosis-related proteins were assessed by western blotting; β-tubulin was used as the loading control.*

Discussion

In this study, we determined cytotoxicity and mode of cell death induced by chalcone glycosides in neuroblastoma cells. Chalcone glycosides were found to exhibit selective cytotoxicity against neuroblastoma cell lines as compared to the normal control cells (Table 1). Compound 6 showed good cytotoxicity against two of the neuroblastoma cell lines (LA-N-1, NB-39).

We especially examined the cell cycle status of NB-39 cells treated with compound 6 by flow cytometry. Compound 6 inhibited cell cycle progression to the S phase, and induced cell cycle arrest at the G0/G1 phase. The regulatory proteins that play key roles in controlling cell cycle progression are the cyclins and CDK [7]. Cyclin D and cyclin E, as vital regulatory factors of progression to the G1-S phase of the cell cycle, are known to be involved in the carcinogenesis in most human cancers [20,21]. We then examined the expression levels of the cell cycle-related proteins involved in the G0/G1 and S phases. Expression of cyclin D decreased after 48 h, with induction of cell cycle arrest in the G0/G1 phase. In addition, the expression of p27 increased after 48 h. p27 is involved in negative regulation of cell proliferation. Cyclin E/CDK2 is inactivated by the binding of p27, resulting in cell cycle arrest [22]. In this study, our findings suggested that inhibition of cyclin E/CDK2 activity was caused by increased expression of p27. Furthermore, we also examined the expression levels of the Rb pathway-related proteins. Cyclin D-CDK4/6 and cyclin E-CDK2 controls phosphorylation of the retinoblastoma protein (Rb) [23]. Rb protein is an inhibitor of progression from the G1 to the S phase of the cell cycle. It interacts with a transcription

factor (E2F family) to repress gene transcription for transition [24]. Cyclin D/E-CDK activates the phosphorylation of Rb, which results in the activation of E2F [25,26]. E2F activation leads to transcriptional activation of E2F-responsive genes, and promotes progression of the cell cycle [27,28]. Miscegenation of E2F function is frequently found in cancer, which further supports the role of G1-S transcription in oncogenesis [29]. In this study, we showed that compound 6 induced significant decrease in the activities? of phospho-Rb and E2F-1 in a time-dependent manner. Our findings suggested that compound 6 inhibited progression of the cell cycle to the S phase by inhibiting Rb phosphorylation and release of E2F, inducing G0/G1 phase arrest.

In addition, examination of the NB-39 cells by Hoechst 33342 staining revealed apoptosis, characterized by cell shrinkage, nuclear chromatin condensation, and nuclear fragmentation, in the NB-39 cells treated with compound 6. Flow-cytometric analysis after annexin V-PI double staining also revealed an increase in the number of early apoptotic cells. These results indicate that compound 6 induced apoptosis in the NB-39 cells. Then, we examined the expression levels of the apoptosis-related proteins. Decrease in the expression levels of the executioner caspases (caspases-3, -7), and increase in the cleavage of the same caspases were observed at 72 h. Caspases are known to play broad roles in apoptosis and inflammation. In the pathway of apoptosis, cell death is induced by the activities of the executioner caspases [30]. Moreover, significant decreases in the expression levels of XIAP and survivin were observed following exposure of the neuroblastoma cells to compound 6. Survivin is one of the members of the Inhibitors of Apoptosis Protein (IAP) family proteins, and plays an important role in inhibiting apoptosis [31,32]. Furthermore, the anti-apoptotic role of survivin is known to be mediated by its association with XIAP [33]. PARP is also known to play an important role in the response to DNA damage, inhibition of DNA repair and cell death, and it is cleaved by an effector caspase during apoptosis [34,35]. In this study, we showed that compound 6 induced cleavage of PARP. Therefore, compound 6 may induce apoptosis in neuroblastoma cells *via* a caspase-dependent pathway.

Conclusion

In conclusion, we found in this study that 4'-O-β-d-glucopyranosyl-4-hydroxy-3,3',5-trimethoxychalcone (compound 6) exhibits potent and selective antitumor effect against neuroblastoma cells. It induces cell cycle arrest in the G1 phase by inhibiting S phase transition *via* inhibition of Rb phosphorylation and release of E2F. In addition, compound 6 induces apoptotic cell death, mediated *via* a caspase pathway. These findings indicate that compound 6 may represent a lead compound for the development of a therapeutic drug for neuroblastoma.

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