Inhibition of WNT signaling reduces differentiation and induces sensitivity to doxorubicin in human malignant neuroblastoma SH-SY5Y cells

Junjira Suebsoonthron, Thiranut Jaroonwitchawan, Montarop Yamabhai and Parinya Noisa

Neuroblastoma is one of the most common cancers in infancy, arising from the neuroblasts during embryonic development. This cancer is difficult to treat and resistance to chemotherapy is often found; therefore, clinical trials of novel therapeutic approaches, such as targeted-cancer signaling, could be an alternative for a better treatment. WNT signaling plays significant roles in the survival, proliferation, and differentiation of human neuroblastoma. In this report, WNT signaling of a malignant human neuroblastoma cell line, SH-SY5Y cells, was inhibited by XAV939, a specific inhibitor of the Tankyrase enzyme. XAV939 treatment led to the reduction of β-catenin within the cells, confirming its inhibitory effect of WNT. The inhibition of WNT signaling by XAV939 did not affect cell morphology, survival, and proliferation; however, the differentiation and sensitivity to anticancer drugs of human neuroblastoma cells were altered. The treatment of XAV939 resulted in the downregulation of mature neuronal markers, including *β-tubulin III*, *PHOX2A*, and *PHOX2B*, whereas neural progenitor markers (*PAX6*, *TFAP2α*, and *SLUG*) were upregulated. In addition, the combination of XAV939 significantly enhanced the sensitivity of SH-SY5Y and IMR-32 cells to doxorubicin in both 2D and 3D culture systems. Microarray gene expression profiling suggested numbers of candidate target genes of WNT inhibition by XAV939, in particular, *p21*, *p53*, *ubiquitin C*, *ZBED8*, *MDM2*, *CASP3*, and *FZD1*, and this explained the enhanced sensitivity of SH-SY5Y cells to doxorubicin. Altogether, these results proposed that the altered differentiation of human malignant neuroblastoma cells by inhibiting WNT signaling sensitized the cells to anticancer drugs. This approach could thus serve as an effective treatment option for aggressive brain malignancy. *Anti-Cancer Drugs* 28:469–479

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Introduction

Neuroblastoma is an embryonic malignancy and the most common cancer found in infancy. Human neuroblastoma arises from the neuroblasts with an incidence of about 650 cases/year in the USA and over 100 cases/year in the UK [1]. Primary neuroblastoma arises in the adrenal medulla or the sympathetic ganglia along the para–vertebral axis, and migrates to the notochord and dorsal aorta [2]. Although there are several therapeutic approaches, including surgery, chemotherapy, and radiation, the resistance and recurrence of neuroblastoma often occurs, requiring an alternative effective treatment of the disease. The treatment of resistance neuroblastoma depends on many factors, for instance, initial risk factors and treatment history [3]. Cell signaling-targeted therapy could be an alternative for a better effective therapy. Novel therapeutic treatments altering the differentiation of neuroblastoma cells might help to improve the efficiency of chemotherapy for malignant neuroblastoma [4].

Neuroblastoma cell lines, for instance SH-SY5Y and IMR-32 cells, normally contain a side-population of cells, which show stem cell characteristics, and these stem-like cell phenotypes may explain the resistance and recurrence of human neuroblastoma to these conventional therapies [5]. SH-SY5Y cells are human neuroblastoma cells, isolated from a bone marrow biopsy taken from a 4-year-old female with neuroblastoma. SH-SY5Y cells are often used as an in-vitro model of neuronal function and differentiation [6]. The differentiation states of SH-SY5Y cells were based on morphology and neurite length [7]. SH-SY5Y cells can be transformed into mature neurons by several molecules, such as retinoic acid and PPARβ agonists, and these differentiated neuroblastoma cells have been shown to alter their sensitivity to anticancer drugs [8,9]. SH-SY5Y cells present not only adrenergic

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phenotypes but also express dopaminergic markers. This results in the utilization of SH-SY5Y cells to study Parkinson's disease and other neural disorders [6].

Cell signaling pathways, in particular Notch, Hedgehog, and WNT, play key roles in the survival, proliferation, and differentiation of both stem cells and somatic cells [10,11]. During neural development, WNT signaling plays a role in neural crest formation, neuronal differentiation, as well as neurite outgrowth of early-born hippocampus neurons [12]. It was found that, in non-small-cell lung cancer cell lines and primary cancer tissues, WNT1 and WNT2 were overexpressed and the inhibition of WNT1/2 prevented cell growth and induced apoptosis [13]. Aberrant WNT signaling is associated with a wide array of tumor types and plays pivotal roles in the maintenance of cancer stem cells [14,15]. Some small molecules, in particular XAV939, can modulate the level of WNT signaling by inhibiting the regulation of Axin protein. Therefore, XAV939 is a specific inhibitor of WNT signaling and might be used for cancer therapies [16]. In this report, the biological consequences of WNT inhibition by XAV939 on malignant human neuroblastoma SH-SY5Y cells were investigated, including cell survival, proliferation, gene expression, differentiation, and anticancer drug sensitivity. The candidate target genes of WNT inhibition were determined by microarray global gene expression profiling. The findings of this study indicated that targeted-cancer signaling could serve as an effective strategy for the treatment of malignant human neuroblastoma.

Materials and methods

Cell culture

The human neuroblastoma cells, SH-SY5Y and IMR-32, were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, Missouri, USA), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California, USA), 10 000 U penicillin, and 10 mg streptomycin/ml. The cell lines were grown at 37°C in a 95% air and 5% CO_2 humidified incubator [17].

RNA extraction

This procedure was performed according to NucleoSpin Kit manufacturer's protocol (Macherey-Nagel Company Ltd., Duren, Germany). Briefly, the appropriate volume of buffer RLT was added to the cells. One volume of 70% ethanol was added to the lysate and mixed well by pipetting. Then, 700 µl of the sample, including any precipitate, was transferred to an RNeasy Mini spin column, placed in a 2 ml collection tube, and centrifuged for 15 s at 8000*g* or more. RNA concentration was verified using a Nano Drop microvolume spectrophotometer (Thermo Fisher Scientific, Rockford, Illinois, USA) and agarose gel electrophoresis. The RNA samples were maintained at −80°C or subjected immediately to a reverse transcription experiment.

Reverse transcription-PCR

Total RNA of SH-SY5Y cells was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad Inc., California, USA) according to the manufacturer's protocol. Primers for PCR were obtained from Macrogen (Seoul, Korea). cDNA was performed with primers at a 1 µmol/l final concentration using an anneal temperature of 55–57°C. Primer sequences and the expected lengths of the amplified products are listed in the Table 1.

Western blot analysis

Aliquots (50 µg of protein) of cell lysates were centrifuged (14 000*g*, 10 min) and electrophoresed in an 8% SDS–acrylamide gel. Proteins were transferred electrophoretically to nitrocellulose membranes, which were blocked in 5% (w/v) low-fat milk in 20 mmol/l Tris, 500 mmol/l sodium chloride, and 0.1% (w/v) Tween 20 (at pH 7.5) for 1 h, and further incubated with antibodies against β-catenin (catalog no. sc-7199; 1 : 500 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA) or β-actin (catalog no. sc-47778; 1 : 1000 dilution; Santa Cruz Biotechnology) overnight at 4°C before incubation with an horseradish peroxidase-conjugated secondary antibody. Signal detection was performed using an enhanced chemiluminescence kit (Thermo Fisher Scientific).

Table 1 List of the primers used in this study

Genes	Forward	Reverse
β-Actin	TCACCACCACGGCCGAGCG	TCTCCTTCTGCATCCTGTCG
β-Tubulin III	GCTCAGGGGCCTTTGGACATCTCTT	TTTTCACACTCCTTCCGCACCACATC
C-MYC	TACCCTCTCAACGACAGCAG	TCTTGACATTCTCCTCGGTG
MASH1	TCGCACAACCTGCATCTTTA	CTTTTGCACACAAGCTGCAT
Nestin	CAGCTGGCGCACCTCAAGATG	AGGGAAGTTGGGCTCAGGACTGG
NeuroD1	AGCCCTCTGACTGATTGCAC	GTCTATGGGGATCTCGCAGC
N-MYC	CTTCGGTCCAGCTTTCTCAC	GTCCGAGCGTGTTCAATTTT
PAX6	AACAGACACAGCCCTCACAAACA	CGGGAACTTGAACTGGAACTGAC
PHOX2A	CTTGGCCTTCTTTGGATGCG	AGCCCCTCCACTCCTCTAAC
PHOX2B	CGAGCAAGGAAAAGGCACAC	ACGGTCACGTAGAGGAGACA
SLUG	AACAGTATGTGCCTTGGGGG	AAAAGGCACTTGGAAGGGGT
SOX10	TCCAGGCCCACTACAAGAGC	CAATGTCCACGTTGCCGAAG
TFAP2a	TCAAGTACGAGGACTGCGAG	CCTCGATGGCGTGAGGTAAG

Cell viability assay

Cell viability was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay. Cells were seeded into a 96-well plate and 5 µl of 25 mg/ml MTT was added to each well, including one set of wells without cells as a negative control. Cells were incubated for 2 h at 37°C in an incubator before lysis and incubated overnight. The absorbance at 570 nm was measured. The percentage of viable cells was determined by comparison with negative control cells.

Immunocytochemistry

SH-SY5Y cells were grown on a glass coverslip coated with Geltrex (Gibco BRL, Maryland, USA). Cells were washed twice with PBS and fixed in 4% paraformaldehyde. Then, a permeabilization step was performed by incubating the cells with PBS containing 0.1% Triton X-100 for 1 h and incubated with the primary antibody at the appropriate dilution at 4°C overnight. Each well was then washed with PBS and incubated with the secondary antibody before visualization under a fluorescence microscope.

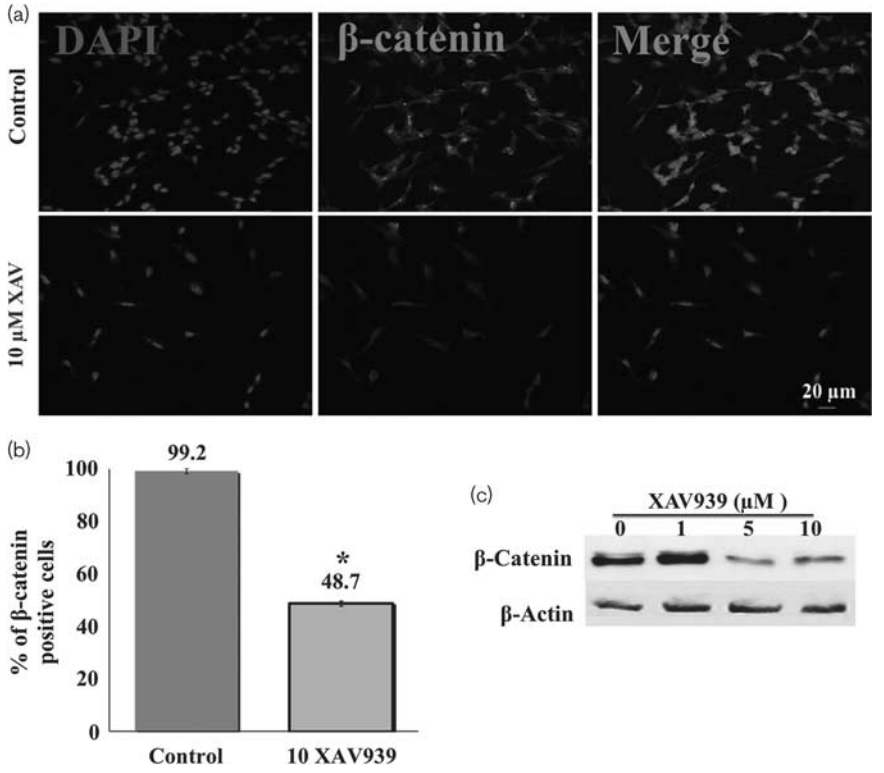
Cell proliferation assay

Cell proliferation was observed by BrdU labeling (DSHB, Iowa, USA). Cells were incubated in media supplemented with 10 µmol/l BrdU for 2 h and washed with PBS. Cells were fixed by 4% paraformaldehyde permeabilized by 2 mol/l HCl at 37°C for 1 h. Anti-BrdU antibody 200 µl (DSHB) was added to each well and incubated at 4°C overnight before adding the secondary antibody and observing under a fluorescence microscope [18].

Anticancer drug sensitivity

The cytotoxicity of doxorubicin to SH-SY5Y and IMR-32 cells was assessed using an MTT assay (Sigma-Aldrich). The experiments were conducted using two groups: (a) cells were treated with doxorubicin alone at 0.001, 0.005, 0.01, 0.05, and 5 µg/ml and (b) cells were treated with similar concentrations of doxorubicin combined with 10 µmol/l XAV939. The optical density was determined by a microplate reader at 570 nm and 630 nm. Average optical density with SDs of duplicate wells were analyzed.

Fig. 1



XAV939 inhibited WNT signaling in SH-SY5Y human neuroblastoma cells. (a) Immunofluorescence of β-catenin in the control and XAV939-treated cells. The fluorescence intensity of β-catenin was reduced on treatment of 10 µmol/l XAV939. (b) The total number of β-catenin-positive cells was significantly decreased when 10 µmol/l XAV939 was added to the culture, from 99.20 to 48.00%, respectively. Data were presented as mean ± SD ($n = 3$). *$P < 0.05$ by Student's *t*-test. (c) Western blot analysis of β-catenin upon treatment of XAV939 at 1, 5, and 10 µmol/l.

Spheroid formation assays

Hanging drop cultures were used to create the spheroid; 20 µl cell suspensions with a seeding density of 5×10^3 cells/drop were placed on the lid of the culture dishes. The bottom of the plate was filled with PBS. Spheroid formation was allowed for 4 days, and then the cell spheroid was transferred to a 24-well plate and exposed to various concentrations of investigated doses. The spheroid migration was observed under an inverted phase-contrast microscope and the relative spheroid migration was assessed on the basis of the diameters of the spheroids measured using the ImageJ software (NIH) program (National Institute of Health, Bethesda, Maryland, USA).

Statistical analysis

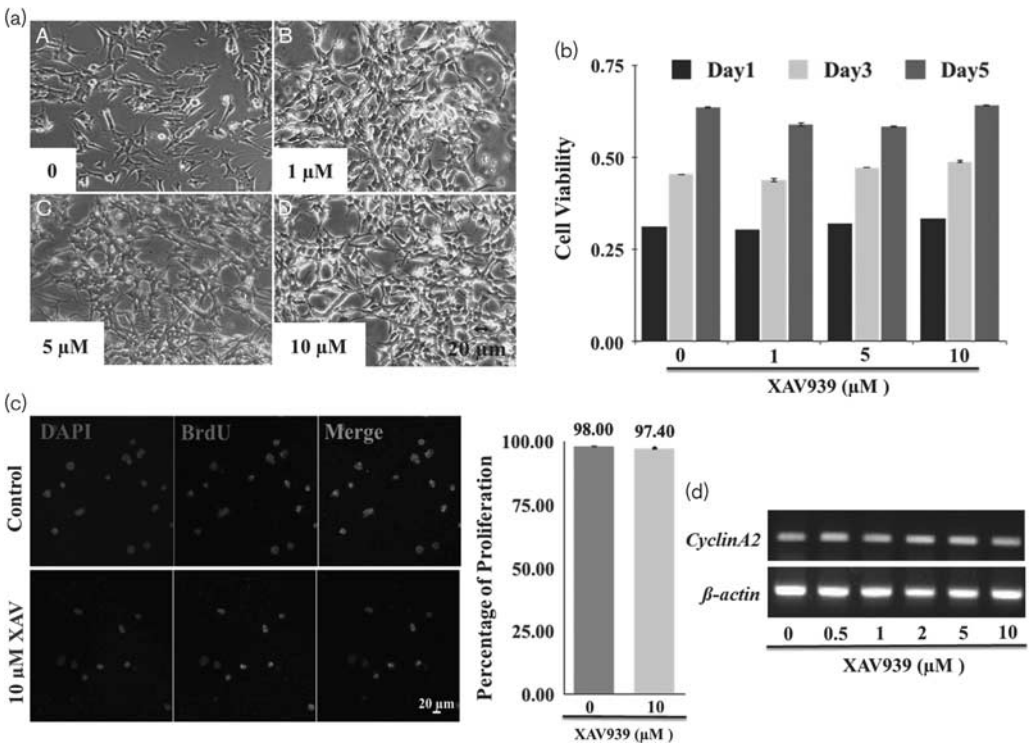
Statistical analyses were carried out by mean ± SD. Student's *t*-test was used to comparing the treatment group and the nontreated group (control). A significant difference was considered at *P* value less than 0.05.

Results

Inhibition of WNT signaling by XAV939 reduced the level of β-catenin in SH-SY5Y cells

XAV939 is a small chemical compound that inhibits WNT signaling through the inhibition of tankyrase enzyme (TNKS1) [16]. To determine whether XAV939 can inhibit WNT signaling and reduce the level of β-catenin of SH-SY5Y cells, immunofluorescence against β-catenin was performed. Upon treatment of 10 µmol/l XAV939 for 24 h, the levels of β-catenin as well as the total number of β-catenin-positive cells were significantly reduced, from 99.21% in the control cells to 48.00% in XAV939-treated cells, and the level of β-catenin inside the nucleus was clearly decreased (Fig. 1a and b). The suppression of WNT by XAV939 was validated by western blotting analysis. XAV939 at various concentrations (1, 5, 10 µmol/l) was added to the cells for 24 h before collecting protein for the analysis. It was found that the level of β-catenin protein was clearly reduced under 5 and 10 µmol/l XAV939 culture conditions (Fig. 1c). This result confirmed that XAV939 inhibited WNT signaling

Fig. 2



Morphology and proliferation of human neuroblastoma cells were not affected by the inhibition of WNT signaling. (a) Cell morphology of SH-SY5Yneuroblastoma did not change upon treatment of XAV939 at indicated concentrations. (b) The inhibition of WNT signaling by XAV939 at various concentrations (1, 5, and 10 µmol/l) did not affect SH-SY5Y cell viability. Cell viability was measured by the MTT assay and reported as OD at wavelength 570 nm. (c) BrdU cell proliferation assays showed no difference between 10 µmol/l XAV939-treated cells and the control cells. DAPI counterstained nucleus and BrdU was recognized by anti-BrdU (DSHB). Data were presented as mean ± SD ($n = 3$). (d) The treatment of XAV939 did not alter the expression of cyclinA2, a cellular mitotic gene. The *β-actin* gene was used as an internal control gene.

by reducing the β-catenin level and its nuclear translocation within human neuroblastoma cells.

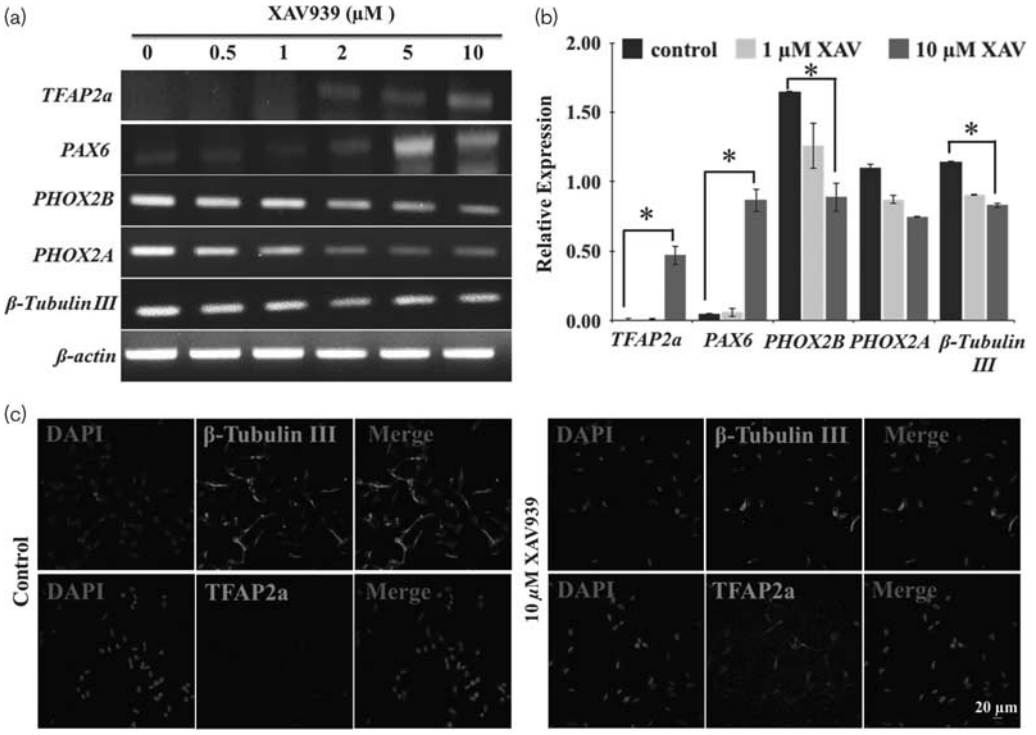
Inhibition of WNT signaling did not alter the cell morphology, viability, and proliferation of SH-SY5Y cells

It has been reported that XAV939 inhibited the proliferation of several cancer cell types, such as colon cancer, by attenuating WNT signaling [16,19]. To validate this effect on human neuroblastoma, SH-SY5Y cells were subjected to MTT cell survival and BrdU cell-proliferating assays. It is noteworthy that upon treatment of XAV939 at various concentrations (1, 5, 10 μmol/l), SH-SY5Y cells did not show alterations in their morphology (Fig. 2a). Cell survival was also not affected by XAV939 as shown by the MTT cell survival assay. SH-SY5Y cells were treated with XAV939 at 1, 5, and 10 μmol/l for 1, 3, and 5 days, and presented a survival rate similar to that of the control cells (Fig. 2b). This result was in line with the BrdU cell-proliferating assay. After the treatment of 10 μmol/l XAV939 for 5 days, there was no significant difference in the number of BrdU-positive cells on comparison between the treated and control groups, 97.40 and 98.00%, respectively (Fig. 2c). The expression of *cyclinA2*, an important mitotic gene, was measured and showed no change in its expression level, either with or without XAV939 treatment (Fig. 2d). This result indicated that the inhibition of WNT signaling by XAV939 did not alter human neuroblastoma cell morphology, viability, and proliferation.

Inhibition of WNT signaling altered the expression of differentiation genes of SH-SY5Y cells

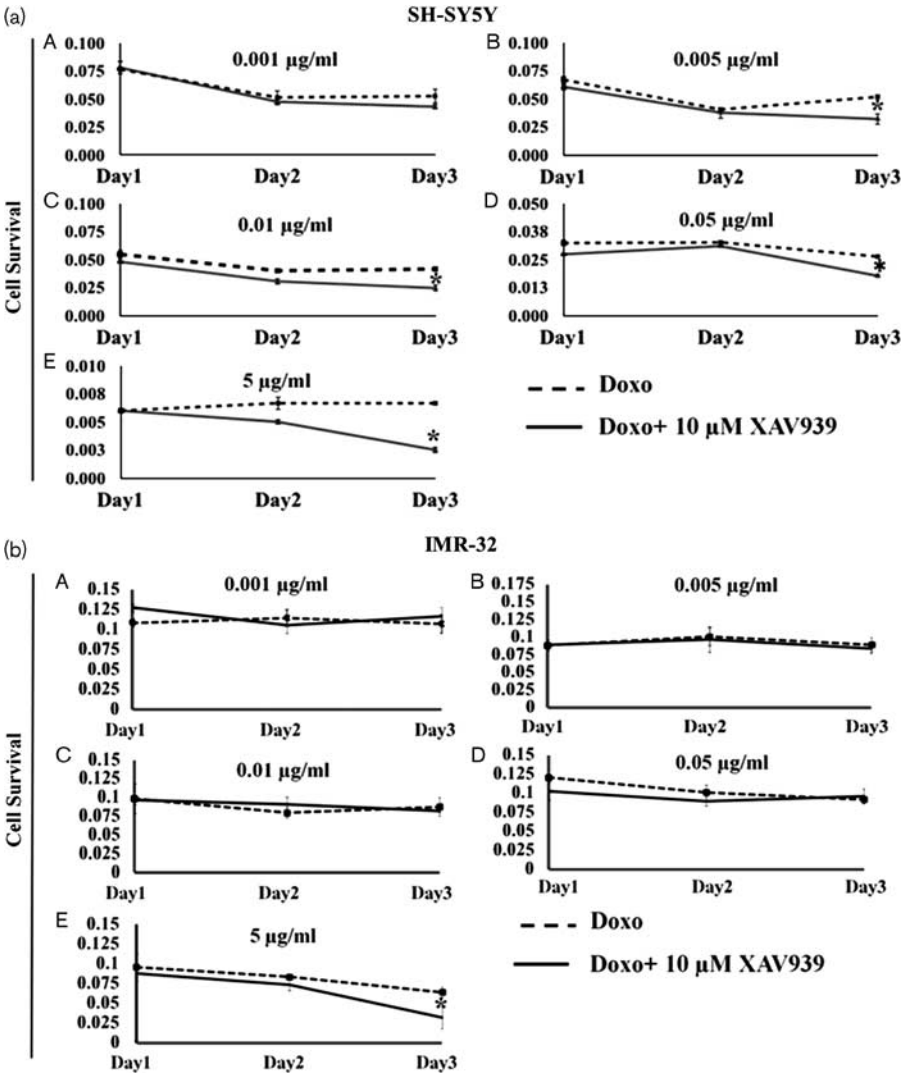
Modulation of the level of WNT signaling has been shown to affect the developmental stages of neural cells [20]. SH-SY5Y cells represent progenitor cancer cell types, whose differentiation could be altered by culture conditions [21]. To examine whether the inhibition of WNT signaling affects the differentiation of SH-SY5Y cells, the expression levels of specific differentiation genes were investigated. Interestingly, it was found that the treatment of XAV939 at 0.5, 1, 2, 5, and 10 μmol/l led to the downregulation of mature neuronal genes, including *β-tubulin III*, *PHOX2A*, and *PHOX2B*. In contrast, the relative expression levels of progenitor markers,

Fig. 3



Inhibition of WNT signaling altered the differentiation of SH-SY5Y human neuroblastoma cells. (a) Gene expressions of differentiated neuronal genes, *PHOX2A*, *PHOX2B*, and *β-tubulin III*, and progenitor markers, *TFAP2a* and *PAX6*, were assessed. The *β-actin* gene was used as an internal control. (b) Upon treatment of XAV939, differentiated neuronal genes were significantly downregulated, whereas progenitor markers were upregulated. Data are presented as mean ± SD ($n = 3$). *$P < 0.05$ by Student's *t*-test. (c) Immunofluorescence confirmed the high expression of β-tubulin III in the control SH-SY5Y cells, whereas TFAP2a protein was detected in XAV939-treated cells. DAPI counterstained nucleus, β-tubulin III, and TFAP2a were recognized by their specific antibodies.

Fig. 4



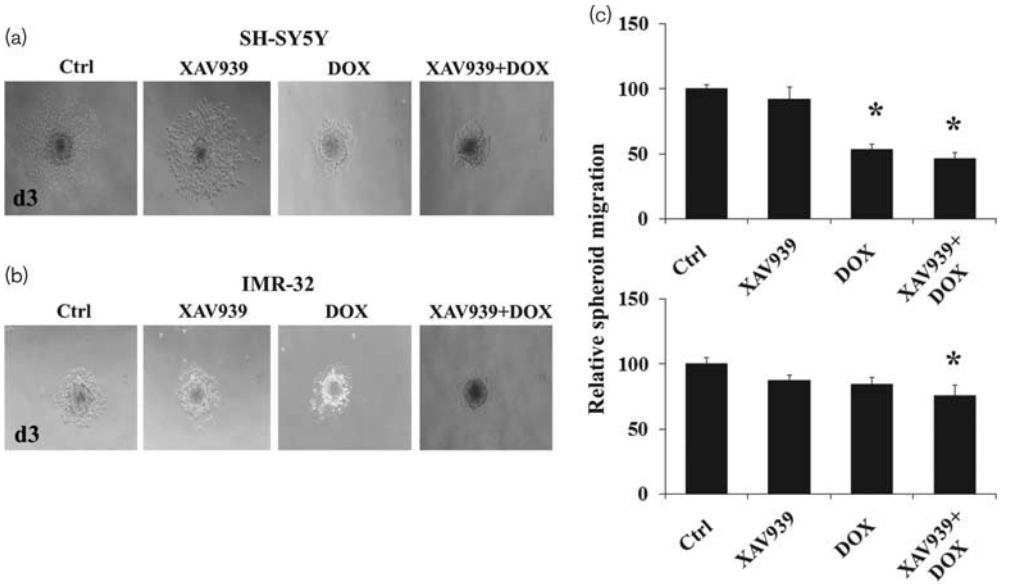
Inhibition of WNT signaling enhanced the sensitivity of SH-SY5Y and IMR-32 cells to an anticancer drug. (a) The sensitivity of SH-SY5Y to doxorubicin was measured by the MTT assay. Doxorubicin at various concentrations [(A) 0.001, (B) 0.005, (C) 0.01, (D) 0.05, and (E) 5 μg/mL] was applied either alone or in combination with 10 μmol/l XAV939. (b) The sensitivity of IMR-32 to doxorubicin was measured by the MTT assay. Doxorubicin at various concentrations [(A) 0.001,(B) 0.005, (C) 0.01, (D) 0.05, and (E) 5 μg/mL] was applied either alone or in combination with 10 μmol/l XAV939. The combination of XAV939 significantly enhanced the anticancer activity of doxorubicin. Data were presented as mean ± SD ($n = 3$). *$P < 0.05$ by Student's *t*-test.

TFAP2a, *SLUG*, and *PAX6*, were significantly upregulated upon treatment of XAV939 compared with the control cells (Fig. 3a and b). In addition, changes in β-tubulin III and TFAP2a, markers of mature neurons and progenitor cells, respectively, were confirmed by immunocytochemistry after the treatment of 10 μmol/l XAV939. The results showed that *β-tubulin III* protein was highly expressed in the control SH-SY5Y cells, whereas the XAV939 treatment induced a clear reduction of *β-tubulin III* (Fig. 3c). However, the expression of TFAP2a protein was undetectable in the control SH-SY5Y, but evidently observed in XAV939-treated cells. These results indicated that the inhibition of WNT signaling reduced the differentiation degree of SH-SY5Y cells.

Inhibition of WNT signaling increased the sensitivity of SH-SY5Y and IMR-32 cells to an anticancer drug

Doxorubicin, a widely used anticancer drug, interacts with DNA by intercalating and inhibiting macromolecular biosynthesis [22]. It also induces histone eviction from open chromosomal areas, leading to the deregulation of transcriptome of cancer cells and, thereafter, attenuating DNA repair, epigenome, and transcriptome [23]. SH-SY5Y and IMR-32 cells are malignant neuroblastoma and resist several anticancer drugs,

Fig. 5



Drug susceptibility test in 3D human neuroblastoma cell models. (a) The morphology of SH-SY5Y cells on 3D spheroid culture after the treatment of different combinations of 5 μg/ml doxorubicin (Dox) and 10 μmol/l XAV939 for 72 h. (b) The morphology of IMR-32 cells on 3D spheroid culture after the treatment of different combinations of 5 μg/ml doxorubicin (Dox) and 10 μmol/l XAV939 for 72 h. (c) The relative spheroid migration of SH-SY5Y and IMR-32 spheroid was observed and measured under an inverted phase-contrast microscope after the cotreatment compared with the untreated control spheroids. Quantitative analysis of spheroid migration was expressed as the relative migration to the untreated control. The values represented the mean ± SD of triplicate experiments. $^*P < 0.05$, compared with the control.

including doxorubicin [24]. The alteration in the differentiation of human neuroblastoma cells could affect their sensitivity to anticancer drugs [25,26]. To determine whether the inhibition of WNT signaling by XAV939 could improve doxorubicin sensitivity of SH-SY5Y and IMR-32 cells, the MTT cell survival assay was performed to measure cell survival. Doxorubicin at various concentrations, 0.001, 0.005, 0.01, 0.05, and 5 μg/ml, was combined with 10 μmol/l XAV939 and applied to the cell cultures. As described previously, the treatment of XAV939 did not cause cell death or proliferation (Fig. 2). Importantly, the addition of XAV939 significantly enhanced the sensitivity of SH-SY5Y cells to doxorubicin at 0.005, 0.01, 0.05, and 5 μg/ml (Fig. 4a). However, IMR-32 showed more aggressive phenotypes than SH-SY5Y cells as only the combined treatment of 10 μmol/l XAV939 and 5 μg/ml doxorubicin resulted in a significant reduction in IMR-32 cell survival (Fig. 4, $P < 0.05$). This resulted showed that the inhibition of WNT signaling improved the sensitivity to anticancer drugs of human neuroblastomas.

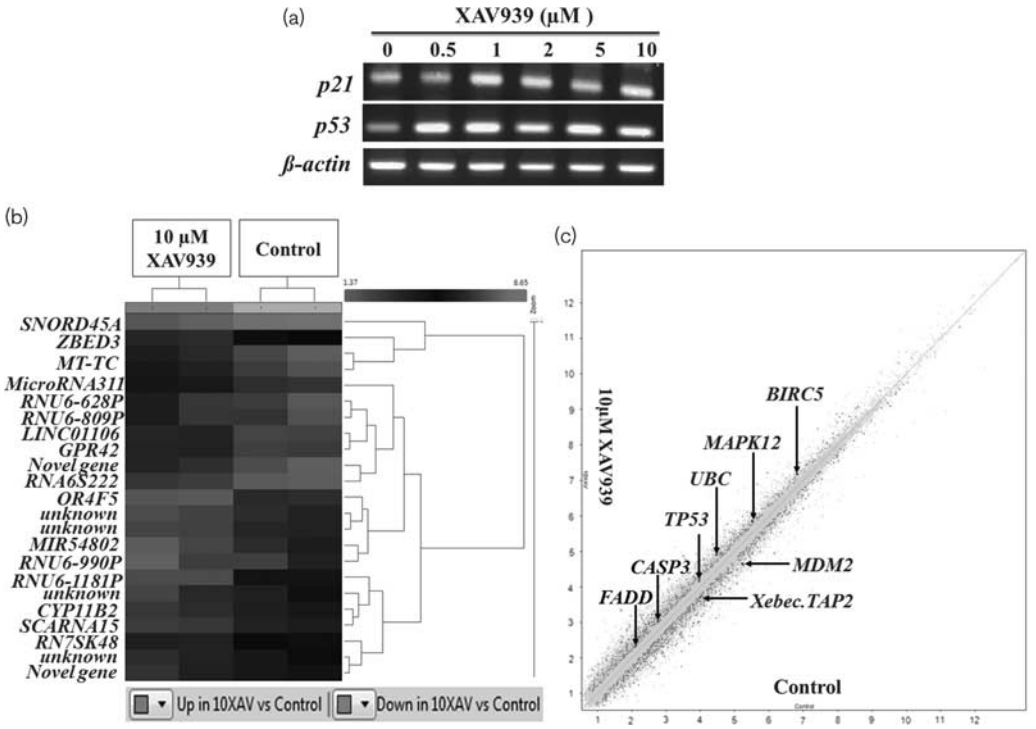
Drug susceptibility test in 3D spheroid models of SH-SY5Y and IMR-32 cells

The chemotherapeutic sensitivity of the cancer cells varies with the microenvironment, especially when moving from a 2D monolayer to a 3D tumor-like environment. Cytotoxicity of the combined treatment, doxorubicin and XAV939, was evaluated in 3D SH-SY5Y and IMR-32 spheroid models, which was prepared by the hanging drop technique to obtain 3D spheroid cells [27]. The relative spheroid migration was observed in a comparison of drug sensitivity toward 3D SH-SY5Y and IMR-32 models upon the treatment of a combined drug. The 3D SH-SY5Y and IMR-32 spheroid migration remained steady when exposed to 10 μmol/l XAV939 alone. Notably, the relative spheroid migration of both NB cell lines was significantly reduced after exposure to the combined treatment of 5 μg/ml Doxorubicin and 10 μmol/l XAV939 at day 3 (Fig. 5a and b). These data suggested that SH-SY5Y and IMR-32 spheroids presented a valid model for drug screening and the anticancer activity of doxorubicin against malignant human neuroblastomas could be enhanced by the inhibition of WNT signaling.

Global gene expression showed the candidate target genes of WNT inhibition that increased doxorubicin sensitivity of SH-SY5Y cells

To further explore the possible factors responsible for the enhanced sensitivity to doxorubicin, the expression levels of the key apoptosis genes, *p21* and *p53*, of SH-SY5Y cells were assessed. It was found that both *p21* and *p53* were upregulated upon the treatment of XAV939 (Fig. 6a). The candidate targets of WNT inhibition of the control and XAV939-treated cells were then determined and compared by the global gene expression microarray. The fold changes of each gene measured in the treated and control SH-SY5Y cells were calculated by measured

Fig. 6



Global gene expression profile showed the candidate targets of WNT inhibition in controlling the anticancer drug sensitivity of SH-SY5Y cells. (a) The expressions of the key apoptosis genes, including *p21* and *p53*, were upregulated in SH-SY5Y cells upon treatment of 10 μmol/l XAV939. The *β-actin* gene was used as an internal control. (b) Global gene expression was shown by microarray. Most differentially expressed genes are shown in the Heatmap. (c) Scattering plot showed that the numbers of cellular apoptotic genes were upregulated by XAV939 treatment, for instance *MDM2*, *CASP3*, *TP53*, and *FADD*.

Table 2 Fold changes in key differentiation genes after the treatment of 10 μmol/l XAV939 versus control cells

Gene level differentiation expression analysis of 10 μmol/l XAV939 vs. control	
Upregulation	
TFAP2a	1.24
Neurotrophin 3	1.24
MYC	1.07
PAX3	1.01
Downregulation	
β-Tubulin III	−1.25
NeuroD1	−1.22
TH	−1.14
GSK3B	−1.09
SOX10	−1.05
β-Catenin	−1.02
PHOX2B	−1.02

intensity and described as a ratio and a change of at least one-fold (up or down) was considered significant. The result reported the comparison of the transcript levels as heatmap and scattering plot of 10 μmol/l XAV939-treated cells and the control (Fig. 6). Compared with the untreated control cells, 23 genes were differentially expressed, of which 15 and eight genes were upregulated and downregulated, respectively (Fig. 6b, paired analysis of variance, $P < 0.05$). Differentiation genes, including *TFAP2a*, *β-tubulin III*, *TH* (tyrosine hydroxylase), *PHOX2B*, and *NeuD1*, were differentially expressed (Table 2), which was consistent with previous gene expression results (Fig. 3). It is noteworthy that a number of apoptosis component genes were upregulated upon the treatment of XAV939, including *ubiquitin C*, *ZBED8*, *FADD*, *CASP3*, *CASP7*, and *MDM2*, explaining the enhanced sensitivity of SH-SY5Y cells to doxorubicin upon the treatment of XAV939 (Tables 2 and 3). The microarray results also showed several microRNAs, transcription factors, and novel transcripts that could provide additional information on the potential novel targets for an effective anticancer therapy. Collectively, the microarray results showed that the inhibition of WNT signaling by XAV939 modulated the expression of differentiation and apoptosis genes that might lead to the enhanced sensitivity of malignant human neuroblastoma cells to anticancer drugs.

Discussion

The WNT pathway plays several roles in both somatic and stem cells, including survival, proliferation, and differentiation [14,28]. It has been acknowledged that WNT signaling serves as a therapeutic target in cancer cells because of its pivotal functions in tumor initiation, tumor growth, and metastasis [29,30]. Aberration of

Table 3 Fold changes of genes related to cell survival, proliferation, apoptosis, and WNT signaling upon treatment of 10 μmol/l XAV939 versus control cells

Genes symbols	Gene name	Fold changes	Annotation
NPHP3-AS1; *UBC;HMG20*, *NPHP3-AS1*	Ubiquitin C	1.35, 1.47	Ubiquitinal-proteasome
ZBED8;Buster3; *C5orf54*	Zinc finger, BED type 8	1.45	Apoptosis pathway
BIRC5	Baculoviral IAP repeat 5	1.28	Apoptosis pathway
CASP3, *CASP7*, *CASP9*		1.22, 1.11, 1.09	Apoptosis/necrosis genes
MAPK12, *MAPK14*, *MAP2K7*, *MAP2K4*	Mitogen-activated protein kinase 12	1.29, 1.07, 1.02, 1.04	Stress activation pathway
PSMC2, *PSMC3*, *PSMC4*, *PSMC5*, *PSMC6*		1.19, 1.05, 1.04, 1.04, 1.58	Proteasome 26s degrade
TP53TG3; *TP53TG3C*; *TP53TG3B*	TP53 target 3; 3C; 3B	1.17	DNA-damage response and apoptosis implicated in this doxorubicin-apoptosis pathway
FADD	Fas (TNFRSF6)-associated through the death domain	1.14	Cell death receptor
TP63	p53-related gene	1.14	Apoptotic gene
UBE2D1	Ubiquitin-conjugating enzyme E2D 1	1.07	
NFKB1		1.07	
JUN		1.03	Apoptosis pathway
FZD1,FZD2, *FZD8 and FZD10*	Frizzle receptor genes	−1.08, −1.26, −1.06, −1.38	WNT receptor
LRP 5, *LRP6*		−1.10, −1.06	WNT receptor
DVL1, *DVL2*, *DVL3*		−1.09, −1.11, −1.11	WNT-related molecule
APC		−1.2	WNT-related molecule
MDM2; *RP11-611O2.5*	MDM2 proto-oncogene, E3 ubiquitin protein ligase	−1.46	Apoptotic gene
HLA-A	Major histocompatibility class I, A	−1.38	
DDC	Dopamine gene	−1.23	Oxidation on reactive oxygen species to reduce stress and cell death
XXbac-BPG246D15.9; *TAP2*; *HLA-DOB*	Novel protein, TAP2-HLA-DOB	−1.23	Resistance gene

WNT signaling increased the risk of neuroblastoma even without MYCN-amplification and the inhibition of WNT signaling could restore the chemosensitivity of various cancer models [31,32]. XAV939, a Tankyrase inhibitor, could inhibit WNT signaling by promoting the formation of the destruction complex and lead to β-catenin degradation [16]. This was in line with our finding, showing a reduced level of β-catenin in SH-SY5Y cells upon treatment of XAV939 (Fig. 1). The treatment of XAV939 reportedly led to the inhibition of cancer cell growth [33,34]; however, the survival and proliferation of SH-SY5Y cells were not affected by this WNT inhibitor (Fig. 2).

SH-SY5Y cells are malignant human neuroblastoma and maintain their potential for differentiation [35]. SH-SY5Y cells could be transformed into mature neurons by several factors, for instance retinoic acid, estradiol, and cholesterol [36,37]. Neuronal differentiation of human neuroblastoma was widely induced by retinoic acid, and upon differentiation, and the differentiated neuroblastoma cells could escape cellular apoptosis by the URG4/URGCP pathway [38,39]. Previous reports have shown that the inhibition of WNT signaling promoted cell apoptosis in the neuroblastoma cell line, but did not report the outcomes of cell differentiation [40]. In this study, it was found that the inhibition of the WNT pathway in SH-SY5Y cells did not affect cell survival and proliferation, but did affect cell differentiation. A number of differentiation genes, such as *PAX6*, *TFAP2a*, *β-tubulin III*, *PHOX2A*, and *PHOX2B*, were altered upon inhibition of WNT signaling (Fig. 3). Importantly, when 10 μmol/l XAV939 was added to the culture, the immunofluorescence results showed a clear reduction of β-tubulin III and increase in TFAP2a protein, indicating that the inhibition of WNT signaling could decrease the expression of mature neuronal proteins and promoted the expression of neural progenitor markers of human neuroblastoma cells. Chemoresistance of human neuroblastoma depends on many factors, including risk factors, genetic background, and treatment history [41]. This issue is one of the challenges for developing a better effective therapy. Signaling-targeted therapy is an upcoming concept for cancer treatment [42,43]. In this study, it was shown that the sensitivity of human neuroblastoma cells to anticancer drug, doxorubicin was enhanced upon the treatment of XAV939 compared with the control. Consistent with other cancer cell types, these results indicated that human neuroblastoma SH-SY5Y and IMR-32 cells were sensitized to anticancer drugs by XAV939 [32,44,45]. A significant decrease in cell survival was found when doxorubicin was cotreated with XAV939 to human neuroblastoma ($P<0.05$) (Fig. 4). This enhanced susceptibility to the anticancer drug of XAV939-treated human neuroblastoma cells was in line with other studies, suggesting that the alteration of differentiation could improve the anticancer drug sensitivity of human neuroblastoma cells [46]. Moreover, because the proposed 3D tumor model bears a close resemblance to in-vivo human cancer tissues, both morphologically and physiologically, the cytotoxicity derived from this

model is more accurate than the cytotoxicity results obtained in 2D monolayer cells [47]. The spheroid cultures of both SH-SY5Y and IMR-32 were more resistant to anticancer drugs than the 2D culture system (Figs 4 and 5). This outcome was believed to be associated with poor drug diffusion through the compact tumor spheroids and the lack of complete bioavailability of the drug to target cancer cells. In addition, a 3D IMR-32 model showed higher chemoresistance than the 3D SH-SY5Y (Fig. 5). However, IMR-32 cells showed a significant dose response to 5 µg/ml doxorubicin and 10 µmol/l XAV939 after 3 days of exposure in both 2D and 3D models. A possible explanation for this might be an aggressive genotype of IMR-32 cells, containing MYCN-amplification, a key driver of malignancy in human neuroblastoma [48,49].

The expression of *p21* and *p53* was upregulated upon treatment of XAV939 in an SH-SY5Y cell culture, proposing that the inhibition of WNT signaling could prime SH-SY5Y cells toward apoptosis (Fig. 4) [40,50]. The global gene expression profile by microarray also explored whether the number of candidate target genes, involved in chemoresistance of neuroblastoma cells, altered their expression level. For instance, the *FZD1* gene, a gene that counteracts anticancer drugs through WNT signaling activation, was upregulated in SH-SY5Y cells upon XAV939 treatment [51]. In addition, apoptosis genes, such as *ubiquitin C*, *ZBED8*, *FADD*, *CASP3*, *CASP7*, and *MDM2*, also showed increased expression by XAV939, and this explained the enhanced anticancer drug sensitivity of SH-SY5Y cells [52].

Altogether, this study showed that the inhibition of WNT signaling led to a reduction of the cellular differentiation stage of human neuroblastoma SH-SY5Y cells. These dedifferentiated SH-SY5Y cells enhanced their sensitivity to anticancer drugs, increasing the expression of cell apoptotic genes. This report highlighted that the modulation of the key cellular signaling pathway, for instance WNT, by a small chemical molecule can enhance the sensitivity of human malignancy to conventional anticancer drugs and suggested a novel molecularly targeted therapy of malignant human neuroblastoma.

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Conflicts of interest

There are no conflicts of interest.

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