Wnt inhibitor XAV939 suppresses the viability of small cell lung cancer NCI-H446 cells and induces apoptosis

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Abstract. Small cell lung cancer (SCLC) is the most aggressive type of lung cancer due to a fast tumor doubling time and early hematogenous spread. Advances in the treatment of non-small cell lung cancer using targeted therapies having been made, but no targeted drugs for SCLC have been approved. The Wnt signaling pathway is associated with tumor progression and metastasis; therefore, the inhibition of Wnt/β-catenin signaling is a strategy for anticancer drugs. Tankyrase 1 (TNKS1) is overexpressed in a number of types of cancer and XAV939 is a small molecule inhibitor of TNKS1 which may inhibit tumor growth. The present study aimed to investigate the potential molecular mechanisms underlying XAV939-induced suppression of the viability of SCLC cells. MTT assays were used to determine the viability-inhibition rate of cells and to identify the drug concentration which optimally inhibited cell viability. Flow cytometry was used to determine whether XAV939 induced apoptosis of SCLC cells, and to analyze the effect of the drug on the cell cycle. The results of the present study identified that XAV939 inhibited the viability of NCI-H446 cells in a dose-dependent manner, but cisplatin inhibited NCI-H446 cell viability in a time- and dose-dependent manner. The combination of XAV939 and cisplatin exhibited a slightly more pronounced inhibition of cell viability at an increased dose of XAV939. In addition, XAV939 markedly induced cell apoptosis of the SCLC cell line H446 by increasing the proportion of cells in the G_0/G_1 phase, leading to inhibition of the cell cycle. The results of the present study indicated that XAV939 inhibited the viability of the NCI-H446 SCLC cell line by inducing cell apoptosis through the Wnt signaling pathway. Therefore, XAV939 may be useful for the treatment of SCLC.

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Introduction

Small cell lung cancer (SCLC) is an aggressive disease that accounts for ~14% of all lung cancers, and there are ~31,000 patients who are diagnosed with SCLC annually in the USA (1). Due to the fast tumor doubling time and early hematogenous spread exhibited by SCLC, the 5-year survival rate remains <5% and the median survival rate is between 7 and 12 months (2,3). Unlike non-small cell lung cancer, in which major advances have been made using targeted therapies, there are no approved targeted drugs for SCLC (1). Therefore, the identification of effective targeted drugs is required.

One potential therapeutic target for lung cancer is the Wnt signaling pathway (4-7). Wnt signaling regulates cell proliferation, survival, and differentiation, and serves key functions in embryonic development and tumorigenesis (8-11). In the canonical Wnt signaling pathway, glycogen synthase kinase-3, in complex with axin and adenomatous polyposis coli, constitutively phosphorylates β-catenin, maintaining it at a decreased level (12). Poly-ADP-ribose polymerase (PARP) enzymes regulate the canonical Wnt activity: Tankyrase (TNKS) 1 and TNKS2. These two enzymes poly-ADP-ribosylate and destabilize axin, a key component of the β-catenin phosphorylation complex (12-14). TNKS1 has been identified to be upregulated in a variety of types of cancer, including plasma cell leukemia, high-grade non-Hodgkin's lymphoma, breast, colon and bladder cancer (15-20).

XAV939 is a small molecule TNKS inhibitor and is synthetized using a chemical genetics approach (21). Waaler *et al* (22) and Bilir *et al* (23) revealed that XAV939 suppressed the viability of colon cancer cells and triple-negative breast cancer cells by inhibiting Wnt signaling. However, the association between SCLC and the Wnt signaling pathway remains unknown. To the best of our knowledge, it has not been identified whether XAV939 exhibits an effect on SCLC cells, and it is hypothesized that the underlying molecular mechanism may contribute to establishing SCLC targeted therapy.

In the present study, the Wnt pathway inhibitor XAV939 was investigated in the effective treatment of SCLC cells and the inhibitory effect of XAV939 on the viability of SCLC cells was identified. In addition, the effect of XAV939 on the cell cycle and cell apoptosis was determined. The results of the present study revealed that XAV939 may inhibit the viability of SCLC via the repression of TNKS1, and TNKS1 may be a target for eliminating SCLC cells.

Materials and methods

Chemicals and reagents. XAV939, MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The cisplatin injection was purchased from Hospira Australia Pty, Ltd. (Melbourne, Australia). The cell cycle detection kit and annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Nanjing KeyGEN Biotech. Co. Ltd. (Nanjing, China).

Cell culture. The NCI-H446 human SCLC cell line was purchased from the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China). Cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all purchased from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) under standard cell culture conditions (37˚C, 100% relative humidity, atmosphere containing 5% CO_2).

MTT cell viability assays. To investigate the effectiveness of XAV939 targeting the Wnt signaling pathway in SCLC cells, the inhibitory effects XAV939 on the viability of H446 cells was determined. Cell viability was measured using an MTT colorimetric dye reduction assay, as previously described (24). The assays were divided into three groups and each group received various drug concentrations, as follows: XAV939 group (2, 4, 8, 16 and 32 μM XAV939), cisplatin group (1, 2, 4, 8 and 10 mg/l cisplatin) and combination group (2.0 mg/l cisplatin combined with 2, 4, 8, 16 or 32 μM XAV939). Each experiment was performed in 96-well plates and repeated three times. The concentration range for treatment with each inhibitor was determined on the basis of previous studies (12,25). A total of $1x10^5$ NCI-H446 cells/well were seeded in 96-well plates and treated with the three groups drugs following incubation for 24 h. Cells treated with the drugs were exposed for 24 or 48 h at 37˚C, following which the drug was removed. A total of 100 μl MTT was added to each well and incubated for 4 h. Subsequently, the medium was removed and 100 μl DMSO was added to dissolve the solid formazan for 15 min. The absorbance at a wavelength of 570 nm was then determined.

Apoptosis analysis. Apoptosis was determined using an annexin V/FITC apoptosis detection kit, according to the manufacturer's protocol. NCI-H446 cells ($1x10^6$ cells/ml) were seeded into 6-well plates and subsequently treated with PBS (control) and XAV939 (8, 16 and 32 μM) at 37˚C for 24 h. Cells were re-suspended in 500 μl 1X binding buffer and incubated with Annexin V/FITC and propidium iodide (PI) for 20 min at room temperature in the dark. Cells were analyzed using fluorescence-activated cell sorting (FACSCalibur; BD Biosciences, San Jose, CA, USA) and FlowJo software (version 7.6; Tree Star Inc., Ashland, OR, USA).

Cell cycle analysis. Cell cycle was determined using a cell cycle detection kit (Nanjing KeyGEN Biotech. Co. Ltd.), according to the manufacturer's protocol. The assays were divided into two groups: PBS control group and XAV939 group. NCI-H446 cells with a concentration of $1x10^6$/ml were seeded into 6-well plates and treated with PBS (control), XAV939 (8, 16 and 32 μM) at 37˚C for 24 h. Cells were harvested and fixed with 70% ethanol at 4˚C for 12 h and subsequently incubated at 37˚C for 30 min with 100 μl RNase A. A total of 400 μl PI was added to the cells at 4˚C in the dark for 30 min. Cell cycle distribution was analyzed for 10,000 selected cells using the Aria II flow cytometer (BD Biosciences) at 488 nm wavelength. The resulting DNA distributions were analyzed using FlowJo software (version 7.6; Tree Star Inc.) to determine the proportion of cells in G_0/G_1, S and G_2/M phases of the cell cycle.

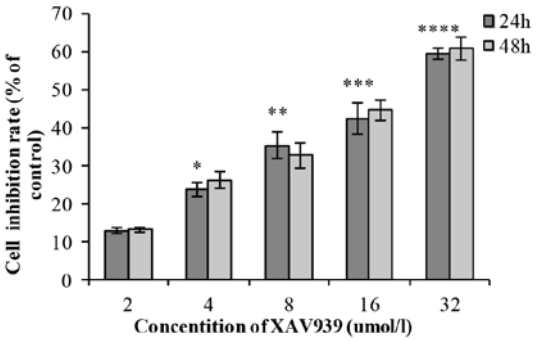

Figure 1. Cytotoxic effect of XAV939 on NCI-H446 cells. The viability of NCI-H446 cells incubated with 2, 4, 8, 16 and 32 μM XAV939 for 24 and 48 h was assessed by MTT assay. Significant differences were observed in a concentration-dependent manner; however, there were no statistical significance differences as time increased. *P<0.01 vs. 2 μM; **P<0.01 vs. 4 μM; ***P<0.01 vs. 8 μM; ****P<0.01 vs. 16 μM.

Statistical analysis. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Multiple group comparisons were made using one-way analysis of variance and the post-hoc tests were performed using LSD test and Dunnett t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

XAV939 effectively inhibits the viability of NCI-H446 cells. In the XAV939 group, with the increase of drug concentration (2, 4, 8, 16 and 32 μM), the cell inhibition rate increased gradually and the difference was statistically significant (P<0.01; Table I). As incubation time (24 or 48 h) increased, the cell inhibition rate of each drug concentration increased gradually; however, there was no statistically significant difference between the 24 and 48 h (P>0.05; Table I). The half-maximal inhibitory concentration, IC_{50}, was 20.02 μmol/l. XAV939 induced an inhibitory effect on the viability of NCI-H446 cells in a dose-dependent manner (Fig. 1). Each experiment was performed three times.

Cisplatin inhibits the viability of NCI-H446 cells. Following treatment of cells with 1, 2, 4, 8 or 10 mg/l cisplatin for 24 or 48 h, cell viability decreased in a time- and dose-dependent manner (Fig. 2). The difference was identified to be statistically significant (Table II). The IC_{50} was 2.056 mg/l.

Table I. NCI-H446 cell viability following treatment with XAV939.

	Cell viability (%)		Comparison between treatment durations	
Treatment	24 h	48 h	F	P-value
XAV939, μM				
2	12.99±0.78	13.18±0.63	0.500	0.855
4	23.78±1.83	26.30±2.18	0.135	0.426
8	35.45±3.50	32.72±3.30	0.043	0.602
16	42.50±4.10	44.64±2.69	0.231	0.686
32	59.57±1.45	60.89±3.03	2.985	0.715
Comparison between treatment concentrations				
F	45.027	50.605		
P-value	<0.001[a]	<0.001[a]		

Data are presented as the mean ± standard deviation. [a]P<0.01, [b]P<0.05 vs. control.

Table II. NCI-H446 cell viability following treatment with cisplatin.

	Cell viability (%)		Comparison between treatment durations	
Treatment	24 h	48 h	F	P-value
Cisplatin, mg/l				
1	30.26±2.58	36.61±0.99	4.277	0.084
2	58.03±0.58	64.32±1.10	1.818	0.007[b]
4	64.18±1.02	71.40±0.62	1.837	0.004[b]
8	73.05±0.71	78.47±0.76	0.028	0.007[b]
10	71.98±1.19	82.23±1.09	0.007	0.003[b]
Comparison between treatment concentrations				
F	151.939	107.277		
P-value	<0.001[a]	<0.001[a]		

Data are presented as the mean ± standard deviation. [a]P<0.01, [b]P<0.05 vs. control.

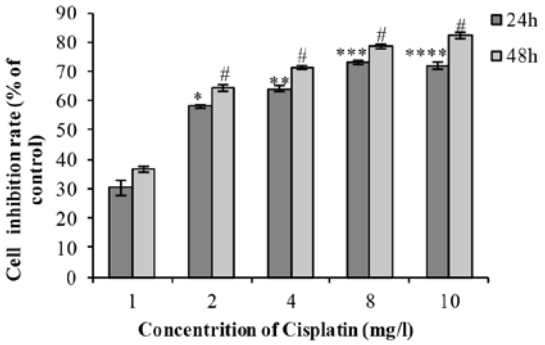

Figure 2. Cytotoxic effect of cisplatin on NCI-H446 cells. Viability of NCI-H446 cells incubated with 1, 2, 4, 8 and 10 mg/l cisplatin for 24 and 48 h. Significant differences were observed in a concentration- and time-dependent manner. *P<0.01 vs. 1 mg/l; **P<0.01 vs. 2 mg/l; ***P<0.01 vs. 4 mg/l; ****P<0.01 vs. 8 mg/l; #P<0.01 vs. 24 h.

Combination treatment inhibits the viability of NCI-H446 cells. In the combination group, the cell inhibition rate increased with the extension of time (24 or 48 h) and with XAV939 concentration (2, 4, 8, 16 or 32 μM). The results of the present study indicated that a combination of the two drugs effectively inhibited NCI-H446 cell viability in a dose- and time-dependent manner (Table III; Fig. 3). When distinct concentrations of XAV939 were combined with cisplatin (2.0 mg/l), the inhibition of NCI-H446 cell viability was slightly decreased at low doses compared with XAV939 treatment alone, and was slightly increased at high doses compared with XAV939 treatment alone (Fig. 4). However, there was no statistically significant difference between combination group and the XAV939 group at any concentration of XAV939.

XAV939 induces NCI-H446 cell apoptosis. To determine whether the inhibitory effect of XAV939 on cell viability was associated with the induction of cell apoptosis, NCI-H446 cells were treated with a variety of concentrations (0, 8, 16 and 32 μM) of XAV939 for 24 h. The proportion of apoptotic

Table III. Cell viability of NCI-H446 cells following combined treatment of XAV939 of the indicated concentrations and 2.0 mg/l cisplatin.

	Cell viability (%)		Comparison between treatment durations	
Treatment	24 h	48 h	F	P-value
XAV939, μM				
2	9.11±0.48	12.62±0.45	0.058	0.006[b]
4	18.66±0.73	23.55±0.56	0.535	0.003[b]
8	33.07±0.56	38.37±0.60	0.001	0.003[b]
16	46.26±1.17	53.81±1.06	0.001	0.008[b]
32	65.22±1.61	70.34±0.38	2.267	0.036[b]
Comparison between treatment concentrations				
F	490.954	1228.98		
P-value	<0.001[a]	<0.001[a]		

Data are presented as the mean ± standard deviation. [a]P<0.01, [b]P<0.05 vs. control.

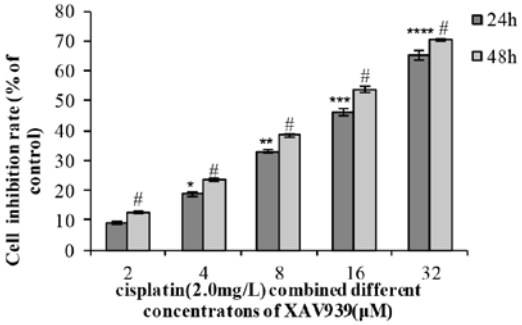

Figure 3. Cytotoxic effect of the combination group on NCI-H446 cells. Viability of NCI-H446 cells incubated with a variety of concentrations (2, 4, 8, 16 or 32 μM) of XAV939 combined with cisplatin (2.0 mg/l) for 24 and 48 h. Significant differences were observed in a concentration- and time-dependent manner. *P<0.01 vs. 2 μM; **P<0.01 vs. 4 μM; ***P<0.01 vs. 8 μM; ****P<0.01 vs. 16 μM; #P<0.05 vs. 24 h.

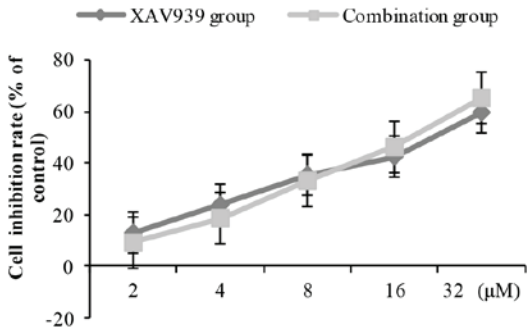

Figure 4. Interactions between XAV939 and cisplatin. When distinct concentrations of XAV939 were combined with cisplatin (2.0 mg/l), the inhibition of NCI-H446 cell viability was slightly weakened at low doses and slightly strengthened at high doses.

NCI-H446 cells increased with XAV939 dosage (P<0.05; Fig. 5), suggesting that the XAV939 may induce increased apoptosis at increased concentrations.

Cell cycle analysis. When the drug group was compared with the control group, the G_0/G_1 phase cell proportion increased significantly, and the S phase to G_2/M ratio markedly decreased. The difference exhibited a statistical significance (P<0.05; Fig. 6). Cell cycle analysis, using flow cytometry, revealed a dose-dependent increase in the accumulation of cells in G_0/G_1 phase (P<0.05; Fig. 6), indicating that XAV939 induced cell apoptosis by cell cycle inhibition.

Discussion

The typical treatment for SCLC is systemic chemotherapy, which exhibits a curative effect; however, the recurrence rate is high, indicating a requirement to develop more effective and targeted therapy options for patients with SCLC. Aberrant activation of Wnt signaling, which serves a key function in the regulation of cell viability, development and differentiation, has been associated with a number of types of cancer including colorectal, prostate, liver, and breast cancer (10,11,26,27). Thus, inhibition of Wnt signaling may be an effective approach to the treatment of distinct types of cancer.

A number of signaling pathway inhibitors have been used to suppress tumor growth. Dickkopf-related protein 1 (28) and secreted frizzled-related proteins (29) are typical Wnt antagonists. XAV939, a novel small molecule Wnt signaling pathway inhibitor, may restrain the abnormal activation of Wnt/β-catenin and exhibit no effect on the normal function of cells. XAV939 may inhibit the activity of tankyrases and may, through poly-ADP-ribosylation, stabilize axin and inhibit Wnt signaling pathways. Compared with other Wnt pathway inhibitors, XAV939 exhibits a strong specificity for the Wnt signaling pathway, without influencing NF-kB or TGF-B signaling pathways.

Huang *et al* (13) and Chen *et al* (14) validated that XAV939 inhibited the viability of colon cancer cells by suppressing Wnt signaling, through binding to the catalytic PARP domain of TNKS. In addition, Bilir *et al* (23) identified that XAV939 inhibited the viability of triple-negative breast cancer cells by

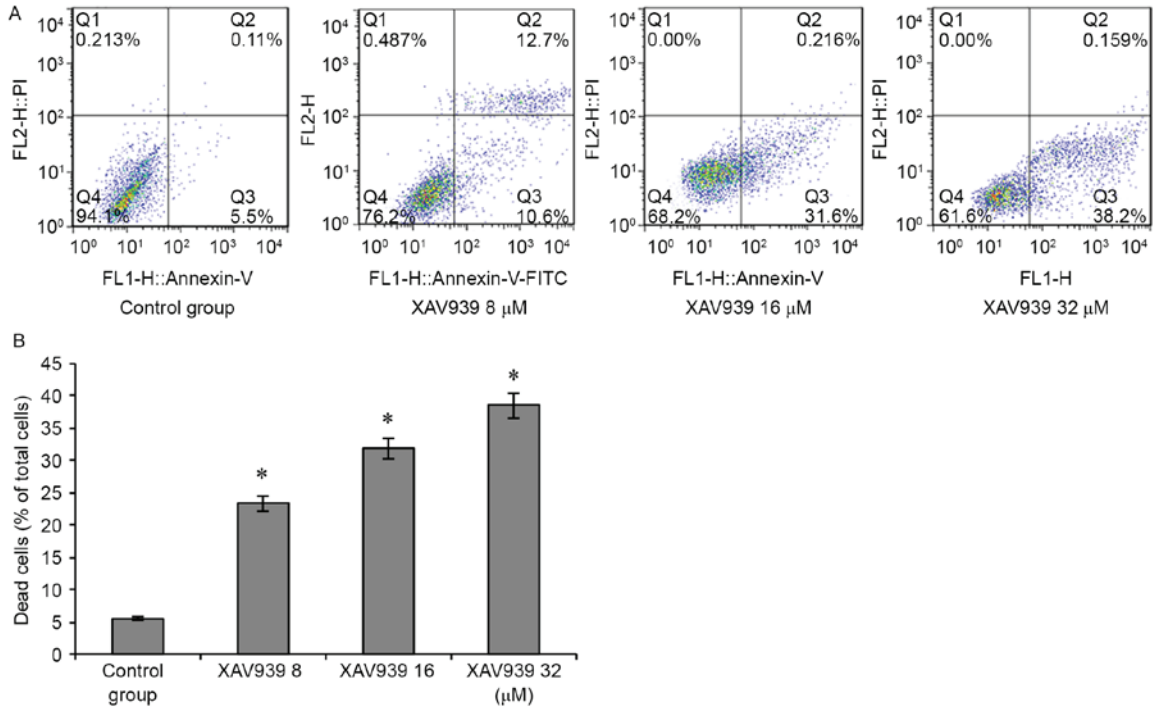

Figure 5. XAV939 induces cell apoptosis in NCI-H446 cells. (A) NCI-H446 cells were incubated with the indicated concentrations of XAV939 for 24 h and cell apoptosis was subsequently analyzed using annexin V and PI double-staining flow cytometry. (B) The proportion of apoptotic NCI-H446 cells increased with XAV939 dosage. *P<0.05 vs. control. PI, propidium iodide; FITC, fluorescein isothiocyanate; FL1-H, cells stained by annexin V; FL2-H, cells stained by PI.

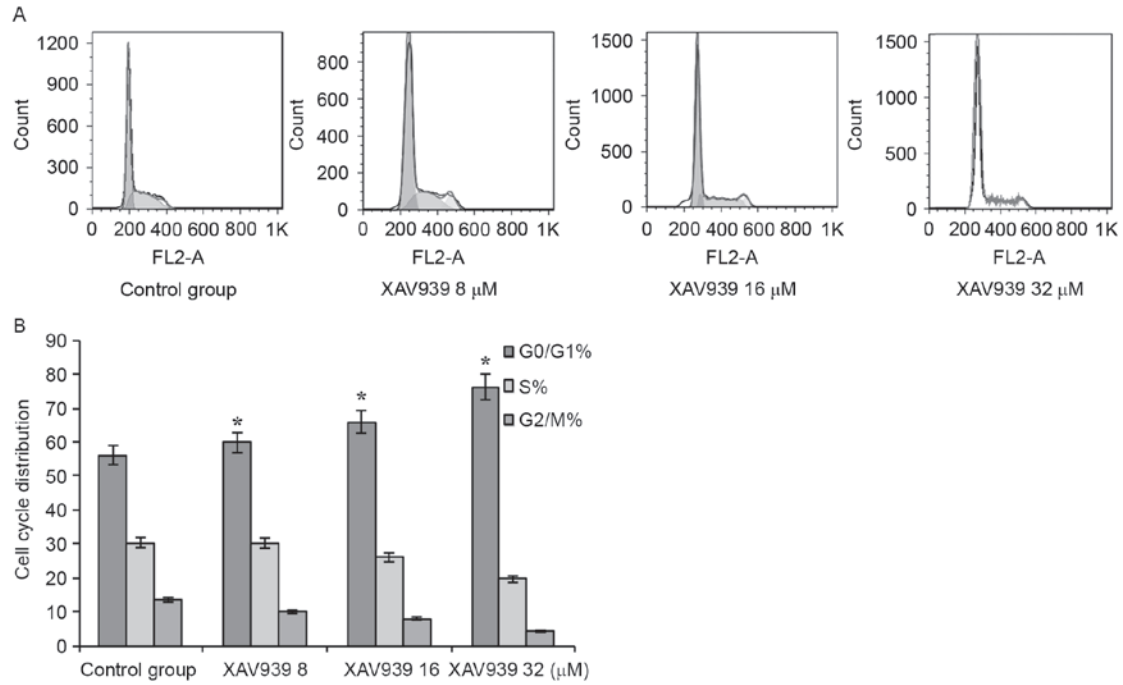

Figure 6. XAV939 induces cell cycle inhibition in NCI-H446 cells. (A) NCI-H446 cells were incubated with distinct concentrations of XAV939 for 24 h, stained with propidium iodide and analyzed for DNA content using flow cytometry. (B) XAV939 induced cell cycle arrest at G_0/G_1 phase in dose-dependent manner. *P<0.05 vs. control. FL2-A, area of fluorescence peak.

suppressing Wnt signaling. Shao *et al* (30) used XAV939 in combination with nedaplatin on HeLa cells, indicating that XAV939 inhibits the viability of cervical cancer cells. To determine whether XAV939 is able to inhibit the viability of SCLC cells, an MTT assay was used. Following treatment with XAV939, a marked inhibition of cell viability in the SCLC cells was observed. When XAV939 was combined with cisplatin, the inhibition of viability of NCI-H446 cells was slightly weakened at a low dose and was slightly strengthened at a high dose compared with XAV939 treatment alone. However, there was no

statistically significant difference between combination group and the XAV939 alone group at any concentration of XAV939.

The effect of XAV939 on the apoptosis of SCLC cells and the cell cycle was analyzed using flow cytometry. Flow cytometry analysis indicated that XAV939 inhibited the viability of NCI-H446 cells. The proportion of apoptotic NCI-H446 cells increased with XAV939 dosage. In addition, cell cycle analysis revealed a dose-dependent increased accumulation of cells in G_0/G_1 phase. The underlying molecular mechanism of apoptosis induction may be as follows: XAV939 decreases the level of β-catenin in the Wnt signaling pathway and prevents it from binding to T cell factor/lymphoid enhancer-binding factor, thus inhibiting the expression of downstream target gene c-myc and Cyclin D1. C-myc gene, an oncogene, primarily functions in the cell cycle and is the switch between G_0/G_1 and S phase. When the Wnt signaling pathway is activated, c-myc gene is expressed, which causes the cells to transition from the quiescent period into the proliferative phase, and promotes cancer cell proliferation, invasion and metastasis (31,32). Cyclin D1 is a specific cyclin that acts on the G_1 phase, which is highly conserved, and promotes dysregulated cell proliferation and malignancy by regulating cyclin-dependent kinase to promote G_1/S phase conversion (33). XAV939 inhibited the expression of c-myc gene and Cyclin D1 by blocking the signal pathway. The cells were arrested in G_0/G_1 phase and induced apoptosis. Furthermore, Yang *et al* (34) identified that XAV939 induced G_0/G_1 phase inhibition in acute lymphoblastic leukemia cells, which is consistent with this study.

The results of the present study preliminary validate XAV939-induced inhibition of cell viability in SCLC, and also provides a reference basis and theoretical support for SCLC targeted therapy. Additional studies on the basis of targeting gene expression to intervene in and inhibit tumor growth, are required. Inhibition of SCLC-associated signaling pathways may provide an effective method of treatment (35).

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