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Nidus vespae protein inhibiting proliferation of HepG2 hepatoma cells through extracellular signal-regulated kinase signaling pathways and inducing G_1 cell cycle arrest

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A protein named NVP(1) was isolated from *Nidus vespae*. The aim of the present study was to elucidate whether and how NVP(1) modulates the proliferation of HepG2 cells. NVP(1) at a concentration of 6.6 μg/ml could arrest the cell cycle at stage G_1 and inhibit the mRNA expression of cyclinB, cyclinD1 and cyclinE. NVP(1) suppressed cdk2 protein expression, but increased p27 and p21 protein expression. However, NVP(1) did not alter p16 protein expression levels. NVP(1) promoted apoptosis in HepG2 cells as indicated by nuclear chromatin condensation, and in addition, the extracellular signal-regulated kinase (ERK) signaling pathway was activated. Moreover, the p-ERK protein expression level was attenuated when the HepG2 cells were pretreated with ERK inhibitor PD98059. These results demonstrate that NVP(1) inhibits proliferation of HepG2 through ERK signaling pathway. NVP(1) could be a potential drug for liver cancer.

Keywords *Nidus vespae*; NVP(1); ERK; cell proliferation

Liver cancer is the most frequent cause of cancer-related death in Asia and is currently being treated with a wide variety of drugs [1]. There are also some non-cancerous diseases in which cellular proliferation is the main characteristic. Several new materials have recently been found to be able to inhibit the proliferation of non-cancerous cells [2,3]. Extracts from *Nidus vespae* can kill bone marrow mononuclear cells and leukemia cells [4,5]. We have been investigating the anti-cell proliferation properties of extracts from *Nidus vespae*, the nest in which one kind of wasp lives in the wild jungle. It is thought that the extracts may act by initiating apoptosis, but little information is available on the molecular targets or the mechanisms by which they exhibit anti-cancer effects.

The cell cycle is regulated through the sequential activation and inactivation of cyclin-dependent protein kinases (Cdk) that control specific steps of the cycle progression, such as G_1-S and G_2-M transitions [6,7]. Cdk activation requires binding to different cyclins, such as cyclinA, cyclinB, cyclinE, and cyclinD, which are expressed during the course of the cell cycle. Cdk activity is also regulated by a diverse family of proteins, termed Cdk inhibitors (Cdki), that bind and inactivate Cdk-cyclin complexes [8–13]. Two classes of Cdki are known, one is the CIP/KIP family, which includes p21, p27, and p57 [13–15], and the other is the INK family, which includes p15, p16, p18, and p19 [16–21]. A central role in the cell cycle progression is played by the retinoblastoma family of proteins, which are critical target substrates, and are phosphorylated by the Cdk-cyclin complexes. The phosphorylation of retinoblastoma proteins controls gene expression through the release of the E2F transcription factor, which can transactivate genes whose products are important for S-phase entry [22]. With regard to the Cdk inhibitors, p16 was not altered by NVP(1) treatment, whereas the expression of p21 and p27 was increased at protein levels.

The mitogen-activated protein kinases (MAPK) family is made up of serine/threonine kinases that comprise three major subgroups, namely extracellular regulated kinase (ERK), p38 MAPK (p38), and C-Jun NH2-terminal kinase

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(JNK) [23]. Of these, ERK is shown to be crucial in cell proliferation and differentiation. Other members of the MAPK family, including p38 and JNK, were originally thought to mediate the cellular stress response and apoptosis [24].

In the present research, we isolated a protein from *Nidus vespae*, named NVP(1), whose apparent molecular mass was 6.6 kDa, and investigated the mechanism of its inhibition of HepG2 cells proliferation. We found that p21, p27 and cdk2 affected the cell cycle in HepG2 cells treated with NVP(1). Our results indicate that the ERK pathway is required for NVP(1) induced growth arrest and antiproliferation response. Therefore, NVP(1) could be a potential drug for liver cancer.

Materials and Methods

Materials

Nidus vespae was obtained from the wild in Hubei province, China. Anti-actin (AC-74) antibody was purchased from the Beyotime Institute of Biotechnology (Haimen, China). Anti-ERK (K-23, sc-94), anti-p-ERK (E-4, sc-7383), anti-JNK (FL, sc-572), anti-p-JNK (G-7, sc-6254), anti-p38 (H-147, sc-7149), anti-p-p38 (sc-7973), anti-p21 (F-5, sc-6246), anti-p27 (F-8, sc-1641), anti-p16 (F-12, sc-1661) and anti-cdk2 (M2, sc-163) antibodies were all purchased from Santa Cruz Biotechnologies (Santa Cruz, USA). PD98059 was purchased from Promega (Madison, USA).

Extraction and purification of *Nidus vespae* protein

Nidus vespae (500 g) was extracted with 1000 ml of distilled water at 4 ºC for 48 h, and then filtered through two layers of filter paper. The filtrate was precipitated with saturated ammonium sulfate at 4 ºC, and dialyzed against phosphate-buffered saline (PBS, pH 7.4) at 4ºC for 72 h, using 0.22 μm filtration membrane. The PBS was changed every 4 h. The extracted liquid was freeze-dried (Thermo Savant; Thermo Fisher Scientific, Waltham, USA), and the powder was dissolved in distilled water. Components of the extract were separated by size exclusion chromatography on Sephadex G-50 column (10 cm×125 cm; Pharmacia, New York, USA). The first portion was subjected to reverse-phase high-performance liquid chromatography (HPLC) TSK ODS-120T column (7.8 mm×300 mm, LKB-produkter, AB, Bromma, Sweden) on a Waters (Milford, USA) system with detection at 214 nm [25]. The only peak, named NVP(1), was collected. The relative molecular mass of NVP(1) was estimated to be 6600 Da by mass spectrum (1100 LC/MSD Trap, Agilent Technologies, Santa Clara, USA).

Cell culture

HepG2 hepatoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Gibco fetal bovine serum (FBS; Invitrogen, Carlsbad, USA) in a humidified incubator in an atmosphere of 5% CO_2 in air at 37 ºC. Cell culture medium was supplemented with 100 units/ml penicillin and 250 ng/ml streptomycin. Cells were treated with NVP(1) at concentrations ranging from 0 to 65 μg/ml. The MEK inhibitor PD98059, at 20 μM, was added 1 h before NVP(1) treatment. Cells were then treated with different concentrations of NVP(1) for various times.

Cell counting and cell cycle analysis

Culture wells (6-well cluster dishes, Costar, Sigma Aldrich, St Louis, USA) were inoculated with 3 ml growth medium containing 5×10^4 cells and allowed to attach overnight. Cell proliferation in response to NVP(1) (6.6 μg/ml) was determined. Cell counts were determined on days 1, 2, 3, 4 and 5. Cells from each well were removed by trypsinization and the resultant suspension was counted using a Hemocytometer (Hausser Scientific, Horsham, USA). The cells were serum starved with 10% fetal bovine serum for 24 h to synchronize them in the G_0 phase of cell cycle. Synchronous populations of cells were subsequently treated in the absence or presence of NVP(1) for 24 h. The cells were washed twice with cold PBS and then centrifuged. The pellet was fixed in 75% (*V*/*V*) ethanol for 1 h at 4 ºC, washed once with PBS, and then resuspended in cold propidium iodide (PI) solution (50 μg/ml) containing RNase A (0.1 mg/ml) in PBS (pH 7.4) for 30 min in the dark. Cell cycle phase analysis was performed by flow cytometry using a flow cytometer (Becton, Dicknson and Company, Franklin Lakes, USA) and Becton Dickinson cell quest software.

RNA extraction and semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted (20 μg), and DNase was used to eliminate the contamination of genomic DNA. The PCR primers pairs used for genes amplification were: actin (NM_001100) (432 bp) actin 1, 5′-caggtcatcactatcggcaa-3′; actin 2, 5′-caaagaaagggtgtaaaacgc-3′; cyclinB (NM_031966) (385 bp) cyclinB 1, 5′-tcgcctgagcctattttggt-3′; cyclinB 2, 5′-gcatcttcttgggcacacaa-3′; cyclinE (NM_001238) (366 bp) cyclinE 1, 5′-aaaatcgacaggacgg-cgag-3′; cyclinE 2, 5′-tgccaagtaaaaggtctccc-3′; cyclin D1 (NM_053056) (310 bp) cyclin D1 1, 5′-aatgtgtgcagaagg-aggtc-3′; cyclin D1 2, 5′-ttgagcttgttcaccaggag-3′;

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proliferating cell nuclear antigen (PCNA) (NM_002592) (410 bp) PCNA 1, 5′-gcactcaaggacctcatcaa-3′; and PCNA 2, 5′-atatggctgagatctcggca-3′. After denaturation at 94 ºC for 2 min, PCR was carried out in a DNA thermal cycler (Biometra Thermocycler, Göttingen, Germany) for 30 cycles. Each cycle includes denaturation at 94 ºC for 40 s, annealing at 54 ºC for 40 s, and extension at 72 ºC for 60 s, followed by a final extension at 72 ºC for 5 min. The PCR products were separated on 2% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA), and visualised by ethidium bromide staining using Gel-doc Image (Bio-Rad laboratories, Milan, Italy).

Western blot analysis

HepG2 cells were collected by rubber policeman, washed in PBS, and lysed in ice-cold PBS buffer [0.l M Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), and 0.1% protease inhibitor cocktail and 10 mM phenylmethylsulphonyl fluoride (PMSF)] for 30 min on ice. The lysates were sonicated for 2 s to shear the DNA to reduce its viscosity, followed by centrifugation at 28,100 *g* for 30 min at 4 ºC. The supernatant was collected, and protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Hercules, USA). Twenty micrograms proteins were applied to a 10% SDS polyacrylamide gel and transblotted onto nitrocellulose membranes (Pall, East Hills, USA). After blocking with 5% milk in TBST (25 mM Tris-HCl, pH 7.4, 144 mM NaCl, 0.1% Tween 20) for 1 h, membranes were incubated with the following primary antibodies: anti-p-ERK (1:1000), anti-ERK (1:1000), anti-JNK (1:1000), anti-p-JNK (1:1000), anti-p38 (1:1000), anti-p-p38 (1:1000), anti-actin (1:5000), anti-p21 (1:1000), anti-p27 (1:1000), anti-p16 (1:1000), anti-cdk2 (1:2000) respectively and followed by incubation with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated rabbit anti-rat IgG antibody (1:3000) for 1 h at room temperature. Protein bands were visualized using the enhanced chemiluminescence (ECL) assay kit (super signal west pico chemiluminescent substrate, PIERCE Biotechnology, Thermo Scientific, Rockford, USA). The densitometry analysis of protein bands was carried out using Image J (National Institutes of Health, Bethesda, USA), an open source image manipulation tool widely used for biomedical image processing analysis systems. The density of each band was normalized by β-actin.

Statistical analysis of the data

All data are presented as mean±SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $P<0.05$ was accepted as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments.

Results

Detection of molecular weight of NVP(1)

Molecular weight of NVP(1) was 6600 Da detected by mass spectrometry (data not shown). The result was then confirmed by 15% SDS-PAGE assay [**Fig. 1(A)**].

Inhibition of HepG2 proliferation by NVP(1) *in vitro*

In the absence of NVP(1), 10% fetal bovine serum caused a time dependent increase in cell numbers, whereas the increase in cell number was obviously reduced in the presence of 6.6 μg/ml NVP(1) [**Fig. 1(B)**].

Comparing with serum-treated control cultures, cells treated with 6.6 μg/ml NVP(1) for 48 h showed unique morphological changes. The cells were much smaller in their size with a change in morphology to spherical shape [**Fig. 1C(g)**]. A more profound effect of NVP(1) was seen at a 20 μg/ml NVP(1) treated for 48 h where cells became much smaller in size with significant changes in morphology. Furthermore, the number of cells sharply decreased [**Fig. 1C(i)**]. Following these morphological changes, the cells started detaching from the culture dishes, presumably due to their death.

The IC_{50} of NVP(1) on the assembly of HepG2 cells was assessed by quantifying the cell count in the presence of different concentrations of NVP(1) between 3.47×10^{-4} μg/ml and 65 μg/ml for 3 d. The percentage of inhibition induced by NVP(1) was normalized against controls and plotted against different concentrations of NVP(1) used in the experiment. The IC_{50} was calculated to be 6.6 μg/ml [**Fig. 1(E)**].

The role of NVP(1) in regulating the HepG2 cell cycle

To gain insight into the mechanism of growth inhibitory effects of NVP(1), we assessed cell cycle distribution of HepG2 cells by flow cytometry. Treatment of HepG2 cells with 6.6 μg/ml of NVP(1) for 24h resulted in the increased accumulation of the cells in G_1 phase [**Fig. 1(D)**]. Cultured HepG2 cells were first synchronized and then treated with NVP(1) for 24 h. In controls, 35.4%±2.7% of HepG2 cells mainly progressed into S phase after serum stimulation for 24 h, however, in the presence of NVP(1) treatment, 89.0%±3.5% of HepG2 cells remained in the G_1 phases, with only 10.95% of cells entering S phase [**Fig. 1(D)**].

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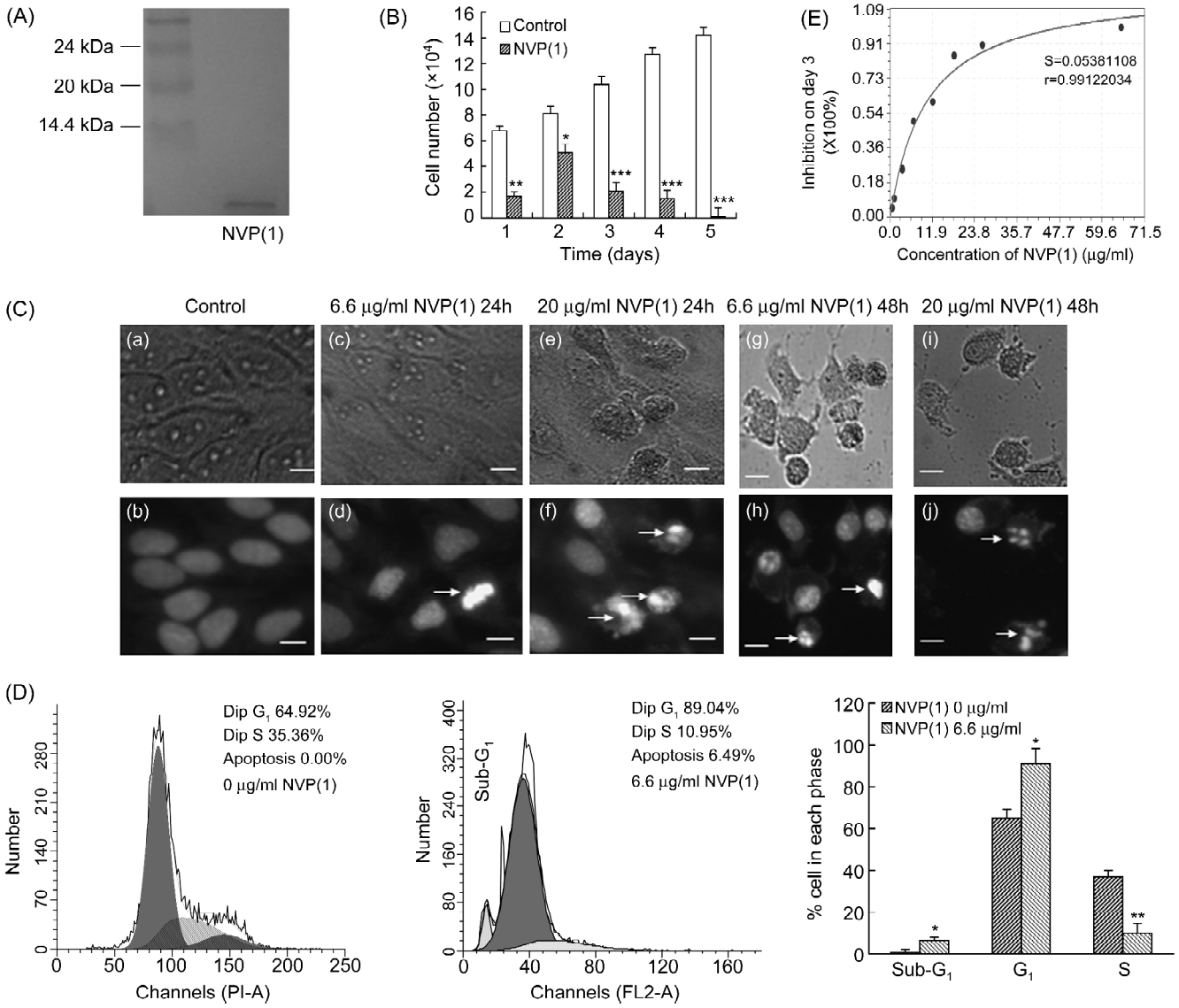

Fig. 1 NVP(1) inhibits serum-stimulated HepG2 proliferation and induces apoptosis (A) Detection of NVP(1) with 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assay. (B) Inhibitory effect of NVP(1) on serum-induced HepG2 growth for 1, 2, 3, 4 or 5 d at 6.6 μg/ml (n=4, performed in triplicate; *P<0.05, **P<0.01, ***P<0.001, compared with control). (C) Effect on cell morphology of HepG2 and induce apoptosis at different concentration of NVP(1) for 24 h and 48 h. In order to determine the mode of cell death induced by NVP (1), morphologic alterations were examined using propidium iodide (PI) DNA staining. Images were taken by phase-contrast [(a), (c), (e), (g) and (i)] and fluorescence [(b), (d), (f), (h) and (j)] microscopy after 24 h or 48 h of NVP(1) or vehicle treatment. In the control group [(A) and (B)], cells are homogeneously stained. In the NVP(1) treatment group [(c), (d), (e), (f), (g), (h), (i), and (j)] nuclear chromatin condensation and presence of granular apoptotic bodies could be observed as indicated by the arrows. Figures are shown from representative pictures. Bar=10 μm. (D), Fluorescence-activated cell sorter analysis of phase distribution in HepG2 treated with 6.6 μg/ml NVP(1) or PBS for 24 h. In the DNA histograms, G_1 and S peaks were observed following 24 h of NVP(1) treatment, sub-G_1 peaks were observed following 24 h of NVP(1) treatment, as indicated by the arrows. Distribution of HepG2 cells at different phases of the cell cycle with or without NVP(1) for 24 h was analyzed by flow cytometry using Cell Quest software. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean±SD of three independent experiments. The significance was determined by Student's t-test (*P<0.05 and **P<0.01 versus untreated control). (E) Curve concentration and effective of HepG2 treated by NVP(1). HepG2 were treated by different concentrations of NVP(1) ranging between 0.0001 and 65 μg/ml. IC_{50} was assessed by plotting the percentage of inhibition on the barrier induced by NVP(1) on day 3 at the time the barrier was established versus controls (y-axis) against different concentrations of NVP (1), which was added to the cultures at time 0 (x-axis). To assess the percentage of inhibition, the following formula was used: [(TERCtrl–TERExpt)/TERCtrl]×100%, where TERExpt represents TER readings in the bicameral units on day 3 where HepG2 cells were treated with NVP(1) at a specified concentration, TERCtrl represents TER readings in the corresponding cultures on day 3 where cells were not exposed to NVP(1). Each data point is the mean±SD of three separate experiments using different batches of cells, and each time point had triplicate cultures.

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On average, the G_1 phase cells treated with NVP(1) was 90.6%±2.0% (n=4). This indicates that the growth suppressive effect of NVP(1) is to arrest the cell cycle at the G_1 phase.

NVP(1) induces apoptosis in HepG2 cells

In the flow cytometric analysis, the observation of sub-G_1 peaks at 24 h following NVP(1) treatment has suggested that NVP(1) could promote apoptosis in HepG2 cells following cycle arrest [**Fig. 1(D)**]. One of the early events of apoptosis is the condensation of nuclear chromatin. We therefore investigated the morphology of NVP(1)-treated cells using PI staining, cells treated 20 μg/ml NVP(1) for 48 h displayed the typical morphology of nuclear chromatin condensation [**Fig. 1C(j)**] when compared with the control cells without NVP(1) treatment [**Fig. 1C(b)**].

NVP(1) decreases cyclins (B, E, and D1) mRNA levels

In order to examine whether NVP(1) could affect cyclins transcription, semi-quantitive RT-PCR were performed using primer pairs specific for cyclinB, cyclinD1, cyclinE, and using actin as a control. It was shown clearly that there were significant decreases in the mRNA level of cyclinB, cyclinD1, and cyclinE in HepG2 cells treated with NVP(1) (**Fig. 2**). Surprisingly, mRNA was almost undetectable after NVP(1), treatment for 20 h. However, mRNA expressions of PCNA (**Fig. 2**) and actin (**Fig. 2**) were not influenced by NVP(1).

Effect of NVP(1) treatment on the expression of Cdk and Cdki in HepG2 cells

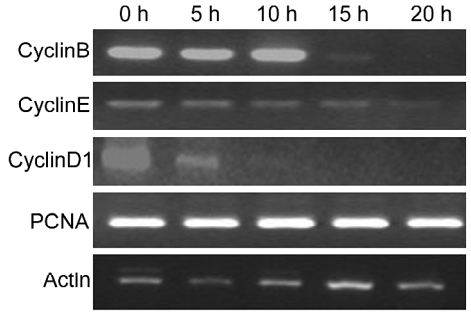

Fig. 2 NVP(1) (6.6 μg/ml) attenuated mRNA expression of cyclinB, E, and D1 by reverse transcription-polymerase chain reaction (RT-PCR) analysis in HepG2 The RNA of HepG2 cells treated by NVP(1) were extracted in the indicated time points (0 h, 5 h, 10 h, 15 h, and 20 h). cDNA was synthesized by reverse transcription to detect cyclin B, E, D1, and PCNA, with actin used as a control. The results are from one representative experiment of three performed that showed similar patterns.

In order to understand the signaling pathways in NVP(1) induced HepG2 cells growth inhibition, specific cell cycle protein mediators were monitored at different time points following NVP(1) treatment by Western blot.

It is known that the cyclin dependent kinase inhibitors p27 and p21 proteins bind and antagonize cyclin/cdks complexes in order to halt cell cycle progression [26]. p27 protein levels remained low level 5 min following NVP (1) treatment, and then sharply increased later (**Fig. 3**). The increase in p21 protein was dramatic 15–120 min following NVP(1) treatment, whereas the p21 protein was not detected in 0–5 min following NVP(1) treatment (**Fig. 3**). No changes in p16 protein were observed in HepG2 cells treated with NVP(1) (**Fig. 3**). Cdk2 proteins are expressed during G_1 phase of the cell cycle and cyclinD1/cdk2, 4 or 6 are required for G_1 progression [27]. In HepG2 cells, Cdk2 protein levels were significantly down-regulated following NVP(1) treatment (**Fig. 3**). The actin protein levels were not changed (**Fig. 3**).

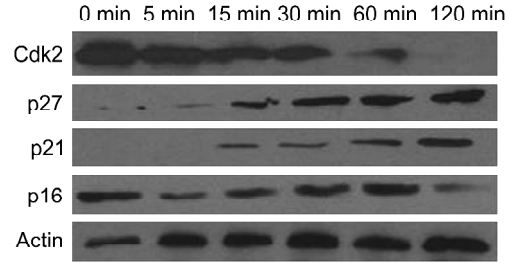

Fig. 3 NVP(1) (6.6 μg/ml) attenuated activation of cdk2 and increased activation of p21 and p27. The cells lysates were prepared and processed to detect cdk2, p21, p27, p16 and actin by Western blotting in the indicated time points (0 min, 5 min, 15 min, 30 min, 60 min, 120 min). The results are from one representative experiment of three performed that showed similar patterns.

Inhibition of HepG2 proliferation by NVP(1) via the activation of the ERK signaling pathway

In order to explore the signaling pathway involved in the inhibition of HepG2 proliferation by NVP(1), we detected the activation of three distinct downstream mitogen-activated protein kinases, ERK, c-Jun N-terminal kinase (JNK), and p38, by measuring the proportion of their phosphorylated forms. HepG2 cells cultured in the presence of NVP(1) were terminated at various times. The levels of total ERK protein remained the same in both control and treated cells [**Fig. 4(A)**]. However, the proportion of phosphorylated ERK increased in a time- and dose-dependent manner [**Fig. 4(B)**]. The total protein levels of

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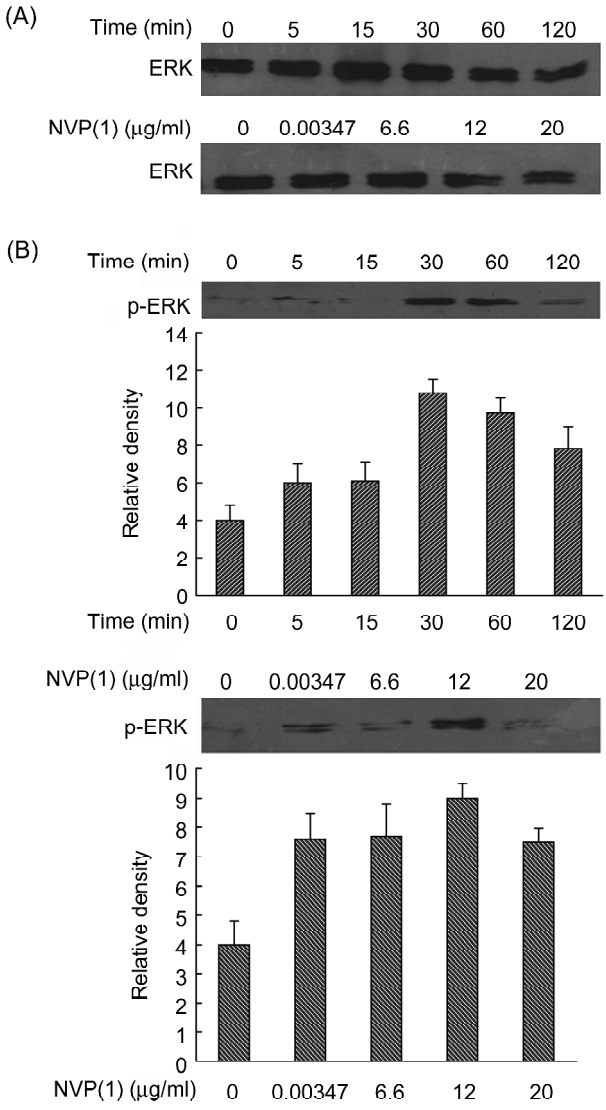

Fig. 4 NVP(1) increased phosphorylation of extracellular signal-related kinase (ERK) activation in a time- and dose-depended manner HepG2 cells were treated by NVP(1) (6.6 μg/ml) in the indicated time points (0 min, 5 min, 15 min, 30 min, 60 min, 120 min). On the other hand, HepG2 cells were treated by four concentrations of NVP(1) for 15 min. The cell lysates were prepared and processed to detect ERK (A) and phosphorylation of ERK1/2 (p-ERK) (B) by Western blotting. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean±SD of three independent experiments.

p38 and JNK were also measured and no changes were observed. The phosphorylated forms of p38 and JNK were not detectable (data not shown). These results demonstrate that NVP(1) inhibits HepG2 cell proliferation through the ERK signaling pathway, whereas the p38 and JNK signaling pathways are not involved.

The MEK1/2 inhibitor blocks phosphorylation of ERK

To confirm NVP(1) inhibiting HepG2 cells proliferation through the ERK signaling pathway, we treated HepG2 cells with PD98059, the potent inhibitor of MEK/ERK, for 1 h before NVP(1) was added to the culture medium for 5, 15, 30, 60 and 120 min respectively. Lysates were used for Western blot analysis. The levels of total ERK protein and actin remained the same in both control and treated cells [**Fig. 5(A)**]. **Fig. 5(B)** shows that in the presence of PD98059 (20 μM), phosphorylated ERK was dramatically reduced. Dimthyl sulfoxide (DMSO) alone had no effect on the level of phosphorylated ERK protein when compared to the control [**Fig. 5(B)**]. The results demonstrated that NVP(1) activated phosphorylation-ERK signal pathway.

Discussion

We detected the extracts from *Nidus vespae* in order to explore whether they can inhibit proliferation of HepG2. First, we isolated one kind of protein designated NVP(1) from *Nidus vespae* to investigate the molecular mechanism of its inhibition on proliferation in HepG2 cells. We found that NVP(1) could suppress HepG2 cell proliferation in both a time- and dose-dependent manner. The same effect was obtained from PC3 prostate cancer cells and primary cultured bronchial smooth muscle cells of wister rats, the results indicating that NVP(1)-inhibited cell proliferation is not selected (data not shown). Additionally, HepG2 treated with NVP(1) was dramatically blocked at the G_1 phase. The results of the Western blot analysis clearly demonstrate that NVP(1) induces cell cycle arrest specifically at the G_1 phase via the up-regulation of p21 and p27 and inhibition of cdk2 protein expression. Moreover, we found that NVP(1) enhanced the ERK activation by phosphorylation.

The importance of cdk2 and cdk4 activation and the subsequent phosphorylation of pRb in the G_1 to S transition have been emphasized in a variety of cells [28]. The phosphorylation of pRb is required to release E2F transcription factors from the pRb/E2F complex. These transcription factors then activate the expression of genes required for initiating DNA synthesis [29]. We investigated the effects of NVP(1) on cell cycle regulatory proteins and their corresponding mRNA. The data demonstrated that cyclinD1, B and E mRNA expression were all attenuated with NVP(1) treatment. By contrast, the proliferating cell nuclear antigen mRNA was not significantly altered (**Fig. 2**). To further elucidate the cell cycle

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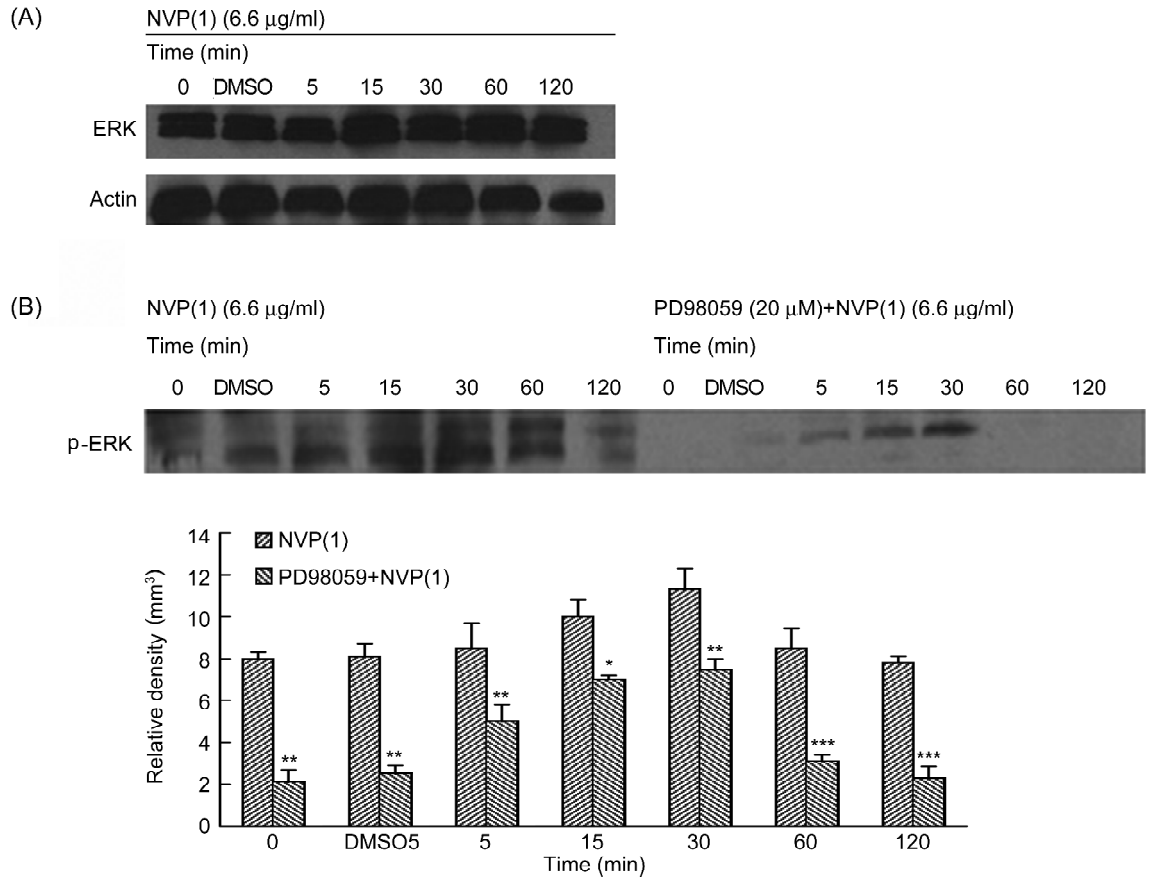

Fig. 5 MEK Inhibitor block phosphorylation of extracellular signal-related kinase (ERK) (A) The cells lysates were prepared and processed to detect ERK by Western blotting in the indicated time points. The same blot was stripped for the detection of total ERK and actin. (B) MEK/ERK inhibitor attenuated phosphorylated ERK (p-ERK) activation. The cells were pretreated with 20 μM of PD98059 for 1h before being stimulated by NVP(1). The lysates were processed for the detection of p-ERK, and the same blot was stripped for the detection of ERK and actin with Dimethyl sulfoxide (0.1%DMSO) and as a control vehicle for inhibitors. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean±SD of three independent experiments. The significance was determined by Student's *t*-test (**P*<0.05, ***P*<0.01, ****P* <0.001, compared with untreated control).

regulation underlying the action of NVP(1), we investigated the involvement of the cycle dependent kinase machinery during the induction of cell cycle arrest. Our data demonstrated a significant down-regulation in cdk2 (**Fig. 3**). A number of studies have shown that the regulation of G_1 cell cycle arrest is probably related with a number of proteins, including other cycle dependent kinase inhibitors, p27, p21 and p16 [30]. However, NVP(1) had no effect on p16 protein level in HepG2 (**Fig. 3**), suggesting that the NVP(1) induced the accumulation of p21 and p27, which could also be responsible for the arrest of the G_1 phase. These results are in agreement with the fact that the cycle dependent kinase and cyclins operate in association with each other by forming complexes, which are inhibited by cycle dependent kinase inhibitor [31]. Therefore, we suggest a series of events in which NVP(1) produces a checkpoint at the G_1-S-phase transition step, thereby resulting in the arrest of HepG2 at the G_1 phase of the cell cycle. As far as we know, this is the first systematic study that demonstrates the involvement of each component of the Cdki-cyclin-Cdk machinery during the cell cycle arrest in HepG2 cells induced by NVP(1). Cycle dependent kinase inhibitor proteins negatively regulate cycle dependent kinase activities. The p21 protein, a member of Kip/Cip family of inhibitor proteins, is known to inhibit the activities of many cyclin/Cdk complexes [32,33]. Our data showed that NVP (1) decreased the cyclin D1 mRNA expression, increased the p21 expression and blocked the cell cycle in the G_1 phase. The results indicate that NVP(1) may act at G_1 or at the G_1-S interface of the cell cycle by modulating cell

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cycle molecule expression, which ultimately leaded to the inhibition of cyclin E-cdk2, cyclinD1-cdk2 and cyclinB-cdk2 complex activity. We have shown that NVP(1) inhibited the passage of cells through the restriction point by decreasing levels of cyclinD1. The restriction point is defined as the time in G_1 when cells continue to progress through the cycle to S-phase. CyclinD1 is one of two rate-limiting cyclins in the passage of cells through G_1 phase of the cell cycle. CyclinD1 levels are low in quiescent (G_0) cells, rise throughout G_1, and remain elevated for the remainder of the cell cycle. CyclinD1 proteins associate with cdk4 and cdk6 kinases [34], and this interaction leads to the activation of their kinase activity. On the other hand, cyclinE associates with cdk2, and this active kinase complex plays a role in the transition from mid-G_1 to S phase of the cell cycle [31]. Collectively, these data demonstrate that NVP(1) induces a G_1 phase cell cycle arrest by decreasing the expression of G_1 phase-specific cyclins (cyclinB, D1, and E) and cdk2. Cycle dependent kinase inhibitors are tumor suppressor proteins that arrest cell cycle progression by binding to active cdk-cyclin complexes thereby inhibiting their activities. The important members of the cycle dependent kinase inhibitor family include p21 and p27, and their expression regulates G_1 phase cycle dependent kinase. Therefore, it is likely that the NVP(1)-mediated induction of p21 and p27 expression is responsible for the decreased Cdk2 kinase activity observed following NVP(1) treatment. In conclusion, the findings presented here demonstrate the anti-HepG2 proliferation effect of NVP(1) *in vitro* is through elevating p21, p27 protein level and inhibiting cyclin D1-cdk2, cyclin E-cdk2 or cyclinB-cdk2 complex activated, leading to DNA synthesis arrested in G1 phase.

Mitogen activated protein kinases signaling cascade pathways comprise a group of three main pathways, including ERK 1 and 2, and two stress-activated protein kinases designated JNK and p38 [35,36]. We analyzed whether the Mitogen activated protein kinase pathway is involved in NVP(1) dependent HepG2 growth inhibit. Among the Mitogen activated protein kinases family, the ERK1/2 cascade is critical to proliferation of many types of cells [37], our data showed that NVP(1) inhibited HepG2 proliferation and increased ERK1/2 activity in a dose- and time-dependent manner. At the same time, we were not being able to detect p-p38 and p-JNK proteins in the HepG2 treated with NVP(1). It is suggested that NVP(1) inhibits HepG2 mitogenesis, in part by increasing ERK1/2 signaling but not through p38 and JNK signal pathways. Therefore, we further examined whether specific inhibition of ERK, PD98059 can attenuate NVP(1)-induced senescence or apopotosis in HepG2. The results [**Fig. 5(B)**] showed that inhibition of p-ERK significantly suppressed NVP(1)-induced HepG2 death.

NVP(1) seems to halt the cell cycle progression until the sub-G1 population increases-a common indication of the presence of apoptosis cells, if this proposition is correct, it implies that HepG2 will start to undergo apoptosis some times after the NVP(1)-induced cell cycle arrest. Many apoptotic bodies were observed by propidium iodide DNA staining when HepG2 cells were treated by NVP(1) for 24 h or 48 h [**Fig. 1(C)**]. At present, NVP(1) is only detected by molecular weight. However, the amino acid sequence and construction for protein have not been elucidated. Moreover, the receptor associated with NVP (1) is also not clear. The experiment presented here testified that NVP(1) promotes apoptosis and inhibits cell proliferation. It is suggested that NVP(1)-induced growth suppression contributes to synthesis effects including apoptosis, cell proliferation, and cell cycle arrest.

In summary, we have demonstrated that NVP(1) prevents HepG2 cell proliferation through activating the ERK signal pathway. On the other hand, NVP(1) arrests the cell cycle at G_1. Finally, the question of whether NVP (1) may act as a regulator of apoptosis or senescence for liver cancer *in vivo* remains to be answered. Our data suggest a possible agent in clinical therapeutic treatment for patients who are suffering from cancer and provide important evidence for understanding the mechanism underlying the inhibitory effect of NVP(1) on HepG2 cell proliferation.

References

1 Paeng JC, Jeong JM, Yoon CJ, Lee YS, Suh YG, Chung JW, Park JH *et al*. Lipiodol solution of ^{188}Re-HDD as a new therapeutic agent for transhepatic arterial embolization in liver cancer: preclinical study in a rabbit liver cancer model. J Nucl Med 2003, 44: 2033–2038

2 Suh SJ, Moon SK, Kim CH. Raphanus sativus and its isothiocyanates inhibit vascular smooth muscle cells proliferation and induce G1 cell cycle arrest. Int Immunopharmacology 2006, 6: 854–861

3 Wang H, Gao X, Zhang B. Tanshinone: an inhibitor of proliferation of vascular smooth muscle cells. Journal of Ethnopharmacology 2005, 99: 93–98

4 Xin XG, Zhang SM, Zhang CY, Shi Y, WU HJ. Effect of the protein from *Nidus Vespae* on the ultrastructure of bone marrow mononuclear cells in acute myeloid leukemia patients. Journal of Chinese Electron Microscopy Society2005, 24: 65–68

5 Susan C, Smolinske PD. Herbal product contamination and toxicity. Journal of Pharmacy Practice 2005, 18: 188–208

6 Schwartz GK, Shah MA. Targeting the cell cycle: a new approach to cancer therapy. J Clin Onco 2005, 23: 9408–9421

7 Bryant P, Zheng Q, Pumiglia K. Focal adhesion kinase controls cellular levels of $p^{27/Kip1}$ and $p^{21/Cip1}$ through Skp2-dependent and

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-independent mechanisms. Mol Cell Biol 2006, 26: 4201–4213
8 Gesbert F, Sellers WR, Signoretti S, Loda M, Griffin JD. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ through the phosphatidylinositol 3-kinase/AKT pathway. J Biol Chem 2000, 275: 39223–39230
9 Gysin S, Lee SH, Dean NM, McMahon M. Pharmacologic inhibition of RAF-MEK-ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of $p27^{Kip1}$. Cancer Res 2005, 65: 4870–4880
10 Croft DR, Olson MF. The Rho GTPase effector ROCK regulates cyclin A, cyclin D1, and $p27^{Kip1}$ levels by distinct mechanisms. Mol Cell Biol 2006, 26: 4612–4627
11 Alt JR, Gladden AB, Diehl JA. $p21^{Cip1}$ promotes cyclin D1 nuclear accumulation via direct inhibition of nuclear export. J Biol Chem 2002, 277: 8517–8523
12 Dupont J, Karas M, LeRoith D. The cyclin-dependent kinase inhibitor $p21^{CIP/WAF}$ is a positive regulator of insulin-like growth factor I-induced cell proliferation in MCF-7 human breast cancer cells. J Biol Chem 2003, 278: 37256–37264
13 Bossenmeyer-Pourié C, Kannan R, Ribieras S, Wendling C, Stoll I, Thim L, Tomasetto C *et al*. The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. J Cell Biol 2002, 157:761–770
14 Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee MH, Massague J *et al*. Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. Nature 1994, 372:570–573
15 Morice WG, Wiederrecht G, Brunn GJ, Siekierka JJ, Abraham RT. Rapamycin inhibition of interleukin-2-dependent $p33^{cdk2}$ and $p34^{cdc2}$ kinase activation in T lymphocytes. J Biol Chem 1993, 268: 22737–22745
16 Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. Circ Res 1995, 76: 412–417
17 Cao W, Mohacsi P, Shorthouse R, Pratt R, Morris RE. Effects of rapamycin on growth factor-stimulated vascular smooth muscle cell DNA synthesis: inhibition of basic fibroblast growth factor and platelet-derived growth factor action and antagonism of rapamycin by FK506. Transplantation 1995, 59: 390–395
18 Abal M, Andreu JM, Barasoain I. Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. Curr Cancer Drug Targets 2003, 3:193–203
19 Héliez C, Baricault L, Barboule N, Valette A. Paclitaxel increases p21 synthesis and accumulation of its AKT-phosphorylated form in the cytoplasm of cancer cells. Oncogene 2003, 22: 3260–3268
20 Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. CyclinE-CDK2 is a regulator of $p27^{Kip1}$. Genes & Dev 1997, 11: 1464–1478
21 Prall OW, Sarcevic B, Musgrove EA, Watts CK, Sutherland RL. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclinD1 expression and decreased cyclin-dependent kinase inhibitor association with cyclinE-Cdk2. J Biol Chem 1997, 272: 10882–10894
22 Cobrinik D. Pocket proteins and cell cycle control. Oncogene 2005, 24: 2796–2809
23 MacCorkle RA, Tan TH. Mitogen-activated protein kinases in cell-cycle control. Cell Biochem Biophys 2005, 43:451–461
24 Kim GS, Hong JS, Kim SW, Koh JM, An CS, Choi JY, Cheng SL. Leptin induces apoptosis via ERK/cPLA2/cytochrome c pathway in human bone marrow stromal cells. J Biol Chem 2003, 278: 21920–21929
25 Agerberth B, Söderling-Barros J, Jörnvall H, Chen ZW, Ostenson CG, Efendić S, Mutt V. Isolation and characterization of a 60-residue intestinal peptide structurally related to the pancreatic secretory type of trypsin inhibitor: Influence on insulin secretion. Proc Natl Acad Sci 1989, 86: 8590–8594
26 Harms C, Albrecht K, Harms U, Seidel K, Hauck L, Baldinger T, Hübner D *et al*. Phosphatidylinositol 3-Akt-kinase-dependent phosphorylation of $p21^{Waf1/Cip1}$ as a novel mechanism of neuroprotection by glucocorticoids. J Neurosci 2007, 27: 4562–7471
27 Morita M, Suzuki T, Nakamura T, Yokoyama K, Miyasaka T, Yamamoto T. Depletion of mammalian CCR4b deadenylase triggers elevation of the $p27^{Kip1}$ mRNA level and impairs cell growth. Mol Cell Biol 2007, 27: 4980–4990
28 Reed SI. Control of the G1/S transition. Cancer Surv 1997, 29: 7–23
29 Seifried LA, Talluri S, Cecchini M, Julian LM, Mymryk JS, Dick FA. pRB-E2F1 complexes are resistant to adenovirus E1A-mediated disruption. J Virol 2008, 82: 4511–4520
30 An FQ, Compitello N, Horwitz E, Sramkoski M, Knudsen ES, Renne R. The Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus modulates cellular gene expression and protects lymphoid cells from p16 INK4A-induced cell cycle arrest. J Biol Chem 2005, 280: 3862–3874
31 Western PS, Miles DC, van den Bergen JA, Burton M, Sinclair AH. Dynamic regulation of mitotic arrest in fetal male germ cells. Stem cells 2008, 26:339–347
32 Encinas M, Tansey MG, Tsui-Pierchala BA, Comella JX, Milbrandt J, Johnson EM Jr. c-Src is required for Glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. J Neurosci 2001, 21: 1464–1472
33 Fabiani R, Rosignoli P, De Bartolomeo A, Fuccelli R, Morozzi G. Inhibition of cell cycle progression by hydroxytyrosol is associated with upregulation of cyclin-dependent protein kinase inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ and with induction of differentiation in HL60 cells. J Nutr 2008, 138: 42–48
34 Ravanko K, Järvinen K, Paasinen-Sohns A, Hölttä E. Loss of $p27^{Kip1}$ from cyclin E/cyclin-dependent kinase (CDK) 2 but not from cyclin D1/CDK4 complexes in cells transformed by polyamine biosynthetic. Cancer Res 2000, 60: 5244–5253
35 Nicole O, Ali C, Docagne F, Plawinski L, MacKenzie ET, Vivien D, Buisson A. Neuroprotection mediated by Glial cell line-derived neurotrophic factor: involvement of a reduction of NMDA-induced calcium influx by the mitogen-activated protein kinase pathway. J Neurosci 2001, 21: 3024–3033
36 Francescato HD, Costa RS, Júnior FB, Coimbra TM. Effect of JNK inhibition on cisplatin-induced renal damage. Nephrol Dial Transplant 2007, 22: 2138–2148
37 Villanueva J, Yung Y, Walker JL, Assoian RK. ERK activity and G1 phase progression: identifying dispensable versus essential activities and primary versus secondary targets. Mol Biol Cell 2007, 18: 1457–1463

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