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To cite this article: Zheng Fu, Li Ren, Huiting Wei, Junqiang Lv, Xuchun Che, Zhifeng Zhu, Jing Jia, Li Wang, Gang Lin, Rong Lu & Zhi Yao (2014) Effects of Tyroserleutide on phosphatidylinositol 3'-kinase/AKT pathway in human hepatocellular carcinoma cell, Journal of Drug Targeting, 22:2, 146-155, DOI: 10.3109/1061186X.2013.844820

To link to this article: http://dx.doi.org/10.3109/1061186X.2013.844820

Published online: 23 Oct 2013.

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Effects of Tyroserleutide on phosphatidylinositol 3'-kinase/AKT pathway in human hepatocellular carcinoma cell

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Abstract
Tyroserleutide (YSL) is an active, low-molecular-weight polypeptide with in vitro and in vivo anticancer effects on human hepatocellular carcinoma BEL-7402 cells. In this study, we studied the effects of YSL on PI3K/AKT in the BEL-7402 cells to explore its anti-tumor mechanism. Results showed that YSL could up-regulate the mRNA and protein expression of tumor suppressor PTEN and increase their activities, meanwhile inhibited the mRNA and protein expression of oncogene AKT and decreased the kinase activities of AKT and PDK1. The resuming balance effect of YSL between PTEN and AKT could prevent the transmission of the three natural amino acids (L-tyrosine, L-serine, and L-leucine) with a corresponding chemical structure of C\textsubscript{18}H\textsubscript{25}N\textsubscript{5}O\textsubscript{6} and molecular weight of 381.42. We previously reported that YSL inhibited hepatocarcinoma growth both in vitro and in vivo [4]. Electron microscopy has shown that YSL destroyed the mitochondrial structure and induces apoptosis in tumor cells [5].

Introduction
Peptides are now considered the new generation of biologically active tools [1]. Besides the well-known peptide families, such as antibiotic macrocyclic peptides, integrin inhibitors, as well as immunoactive, neuromodulator, opioid and hormone peptides, a number of anticancer peptides have been recently reported. As small amino acid sequences, anticancer peptides can be isolated to bind to a predetermined target in the tumor and are potentially capable of interfering with its function. These specific peptides can inhibit individual signaling components, which are essential in cancer development and progression [2,3]. Tyrosyl–seryl–leucine or Tyroserleutide as approved by the Chinese pharmacopoeia committee (YSL), is a tripeptide extracted from pig spleen. The peptide consists of the three natural amino acids (L-tyrosine, L-serine, and L-leucine) with a corresponding chemical structure of C\textsubscript{18}H\textsubscript{25}N\textsubscript{5}O\textsubscript{6} and molecular weight of 381.42. We previously reported that YSL inhibited hepatocarcinoma growth both in vitro and in vivo [4]. Electron microscopy has shown that YSL destroyed the mitochondrial structure and induces apoptosis in tumor cells [5].

Phosphatidylinositol-3 kinase (PI3K) is a heterodimeric enzyme composed of one 110-kDa catalytic subunit and another 85-kDa regulatory subunit and serves as a major signaling component downstream of growth factor receptor tyrosine kinases [6]. PI3K catalyzes the production of the lipid secondary messenger phosphatidylinositol-3,4,5-triphosphate, which in turn activates a wide range of downstream targets, including the serine/threonine kinase AKT [6]. The PI3K/AKT pathway is a critical mediator of multiple cellular processes, including cell proliferation, differentiation, survival, growth, motility and angiogenesis. Studies have shown that the activated PI3K/AKT pathway provides major survival signals to hepatocarcinoma and many other cancer cells [7]. Constitutive activation of AKT is frequently described in many types of human cancers [8]. The objectives of our study were to examine the molecular mechanisms by which YSL regulates the function of proteins in the PI3K/AKT pathway, including PTEN, AKT, PDK1, P21, P27, MDM2, P53 and Bad; and to examine the link between PI3K/AKT pathway and mitochondrial events leading to apoptosis.
Methods

The YSL peptide used in this study was custom manufactured by Shenzhen Kangzhe Pharmaceutical Co. Ltd. (Shenzhen, China). All cell culture media and supplements were purchased from Invitrogen/Gibco (Grand Island, NY). Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. The kits and reagents used in the Real time-PCR were purchased from Applied Biosystems (Foster City, CA) unless otherwise specified. All antibodies were purchased from Cell Signaling Technology (Beverly, MA). MitoProbe Transition Pore Assay Kit and Fluo-4 NW Calcium Assay Kits were purchased from (Beverly, MA). Nucleosome ELISA ANNEXIN V-FITC Apoptosis Detection kit was purchased from BD Biosciences (San Diego, CA). Nucleosome ELISA kits were purchased from Calbiochem (Merck, Germany). PTEN (138G6) antibody, Phospho-PTEN (Ser380/Thr382/383) antibody, Akt antibody, Phospho-Akt (Ser473) antibody, phospho-akt (Thr308) antibody, Phospho-PDK1 (Ser241) antibody, P53 antibody, Phospho-MDM2 (ser166) antibody, Akt antibody, Phospho-Akt (Ser473) antibody, caspase 3 antibody, Bcl-2 antibody, Bcl-XL antibody, P53 antibody, Phospho-Bad (Ser136) antibody, Bcl-2 antibody, Bcl-XL antibody, caspase 3 antibody were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal Anti-P21(WAF/Cip1) Clone CP74, Monoclonal Anti-P27 Kip1 Clone antibody, caspase 3 antibody were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal Anti-P21(WAF/Cip1) Clone CP74, Monoclonal Anti-P27kip1 Clone DCS-72, Monoclonal Anti-beta-Actin Clone AC-15 were purchased from Sigma-Aldrich (St. Louis, MO). Goat antimouse IgG, HRP conjugate and Goat anti-rabbit IgG, HRP conjugate were purchased from Upstate cell signaling solution (Charlottesville, VA). In our previous study [9], in vitro effects of YSL on human hepatocarcinoma cell lines BEL-7402, SMMC-7721, Hep3B, HepG2 and SK-HEP-1 were assayed by the MTS method. Results showed YSL remarkably inhibited the growth of BEL-7402 cells; cell growth was inhibited by 17.7%, 59.9%, 84.3% and 88.5% after 48 h treatment with YSL at 0.4, 0.8, 1.6 and 3.2 mg/ml, respectively. Therefore, we selected BEL-7402 cells line and YSL concentration (1.6 and 0.8 mg/ml). Human hepatocellular carcinoma BEL-7402 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO₂ at 37°C. Cells were seeded in 100-mm cell culture dishes and allowed to adhere overnight. The next day, cells were treated with RPMI-1640 medium (vehicle control) or YSL at concentrations of 0.8 mg/ml or 1.6 mg/ml for 24 or 48 h (n = 3).

Total RNA was extracted and purified using the ToTALLY RNA™ Kit (Invitrogen, Grand Island, NY). First strand cDNA synthesis was performed using 2 μg total RNA, 500 ng oligo(dT)18 and M-MLV Reverse Transcriptase under the conditions recommended by the supplier. The mRNA expression levels of target genes were quantified by quantitative real-time PCR using an ABI Prism 7500 Sequence Detection System (Life Technologies, Grand Island, NY) and SYBR Green Real-Time Master Mix (Toyobo, Osaka, Japan). The Real-time Quantitative PCR for target genes amplification were selected as follows: PTEN FW 5'-TGAGAAGGTTGGAATATCTTCTT-3' and PTEN RW 5'-GTCACTCTTCCATGCAAGTGTTG-3'; AKT1 FW 5'-TCACCCAGATGCAACTCTAGG-3' and AKT1 RW 5'-GGCCACGTAGTCTCTCTTCT-3'; AKT2 FW 5'-GCTCCACAAGCGTGGTAATA-3' and AKT2 RW 5'-CGGAGAAGGTTTAAAGGGG-3'; β-actin FW 5'-TGGCCGACAGGTGCAAGAGA-3' and β-actin RW 5'-AGGGACAGCGGAGGCAGG-3'. The β-actin gene was used as an internal reference, normalizing the amplified target gene cDNA to amplified β-actin in order to compensate for any changes caused by RNA degradation or amplification efficiency. The reaction mixture was incubated at 95°C for 1 min followed by 40 amplification cycles with the following conditions: 15 s at 95°C. 15 s at 57°C and 45 s at 72°C. The fluorescence signal was collected at every extension stage (72°C). The CT value represents the number of cycles for the progression of the fluorescence signal to reach a limitation in each reaction: ΔCT = CT (target gene) – CT (β-actin). The relative expression of a target gene was calculated as 2-ΔCT [10]. Cell protein extracts were prepared by lysing cells for 20 min on ice in a RIPA lysis buffer consisting of 150 mmol/L NaCl, 0.5% Sodium deoxycholate,1% Nonidet P-40, 1% Triton X-100, 1% sodium dodecyl sulfate and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cell debris was removed by centrifugation at 15 000 g for 10 min at 4°C. Then cell extracts were subjected to separation by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after being boiled in Laemmli buffer and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with PBS containing 0.1% Tween 20 and 5% non-fat milk before being incubated with the appropriate primary and secondary antibodies. The bound antibodies were visualized by using LumiGLO Chemiluminescent Substrate Kit (KPL, Gaithersburg, MD).

Following treatment with YSL, BEL-7402 cells were harvested and were detected by flow cytometry for analysis of cell cycle. Briefly, the cells were fixed in cold 70% ethanol at −20°C, and stained with PI solution (70 mmol/l propidium iodide, 38 mmol/l sodium citrate, 20 mg/ml RNase A). The cell cycles of the samples were analyzed by flow cytometry (FASCAlibur, BD, San Jose, CA), and cell distribution into cell cycle phases was calculated using ModFit LT (V3.0) software (Becton, Dickinson and Company, Franklin Lakes, NJ). The mPTP opening was detected with the MitoProbe Transition Pore Assay Kit (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. Briefly, cells were collected and stained with 2 μM calcein AM containing CoCl₂ and ionomycin if required at 37°C for 15 min. Samples were then assessed for fluorescence with excitation at 488 nm by flow cytometry. Different fluorescent signal intensities of subgroup cells were respectively detected, including calcein AM only, calcein AM + CoCl₂, and calcein AM + CoCl₂ + ionomycin. The mPTP opening was indicated by the reduction of mitochondrial calcein signal (difference between calcein AM + CoCl₂ group and AM + CoCl₂ + ionomycin group). YSL-treated cells were trypsinized and resuspended in PBS supplemented with 0.1% BSA and then incubated with Rh123 for 30 min at 37°C. Flow cytometric analysis was then performed to monitor the green fluorescence of Rh123 with excitation at 488 nm by using FASCAlibur (BD Biosciences, San Jose, CA).

The human hepatocarcinoma BEL-7402 cells were seeded at 10 000 cells/well in 96-well black microplate (Poly D-lysin

DOI: 10.3109/1061186X.2013.844820
coated, Greiner Bio-One, Germany) for 24 h. Then BEL-7402 cells were treated with YSL for 24 or 48 h. Intracellular calcium levels of these cells were measured with Fluo-4 NW Calcium Assay Kit (Invitrogen, Grand Island, NY) according to the standard protocol supplied by the manufacturer. The Fluo-4 indicator was excited at 488 nm and analyzed at 525 nm using the Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, Waltham, MA). Measures were expressed as fluorescence intensity. The BEL-7402 cells stained with Fluo-4 indicator were also observed under a fluorescence microscope (Olympus America Inc., Melville, NY). Pictures were captured at the same time. Apoptosis induced by YSL treatment for 24 h was measured using the Annexin V/propidium iodide (PI) detection kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instruction. Briefly, YSL-treated BEL-7402 cells were trypsinized and resuspended in PBS supplemented with 0.1% BSA and labeled with Annexin V-FITC and PI. Flow cytometric analysis was then performed to monitor the green fluorescence of annexin V and the red fluorescence of DNA-bound PI with excitation at 488 nm. All data were collected using a FASCalibur. DNA fragmentation of BEL-7402 cells induced by YSL treatment for 48 h was observed by the method described previously [11]. The quantities of mononucleosomes and oligonucleosomes generated in the apoptotic cardiomyocytes were determined with a quantitative nucleosome ELISA by affinity capturing of free nucleosomes with pre-coated DNA binding proteins. Anti-histone 3 biotin-labeled antibodies were used to detect the levels of nucleosomes with a microplate ELISA reader. This assay was carried out with a commercially available kit from Calbiochem (San Diego, CA). Data were expressed as mean ± SD. Significance was tested using one-way analysis of variance followed by the Student–Newman–Keuls test (SPSS 11.0 software, IBM SPSS, Armonk, NY). Significance was set at p < 0.05.

Results

The ectopic expression of AKT induces cell survival and malignant transformation, whereas the inhibition of AKT activity stimulates apoptosis in a range of mammalian cells [12,13]. AKT has two main isoforms, AKT1 and AKT2, which have both been associated with tumorigenesis [14]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a phospholipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate and inhibits PI3K-dependent activation of AKT [15]. The mutation or loss of PTEN leads to constitutively activated AKT. In this study, treatment with YSL (0.8 or 1.6 mg/ml) significantly reduced the mRNA expression of AKT1 and AKT2 in BEL-7402 cells. Meantime, the mRNA of PTEN in cells treated with YSL increased markedly (p < 0.05) (Figure 1).

AKT is a downstream target of PI3k, the pleckstrin homology domain in the AKT protein binds the products of
PI3k activity, phosphatidyl inositol 3,4,5 phosphate, and is anchored to the plasma membrane where it is phosphorylated and activated by Phosphoinositide-dependent protein kinase 1 (PDK1) and other protein kinases. Full activation of AKT is PI3K-dependent and requires both recruitment to the plasma membrane and phosphorylation on two key residues, Thr308 and Ser47 [16]. Phosphorylation on the activation loop Ser241 by autophosphorylation is necessary for PDK1 activity [17]. Our results showed that YSL inhibited the protein expression of oncogene ATK in heptocarcinoma BEL-7402. To determine the effects of YSL on the activation status of PDK1 and AKT, we evaluated their phosphorylation state in the cancer cell treated with YSL. In all of the YSL-treated groups, phosphorylated PDK1 (ser241) and AKT (Thr308 and Ser473) was significantly lower than those in the control group (Figure 2). As a major negative regulator of the PI3K/AKT signaling pathway, PTEN possesses a carboxy-terminal, non-catalytic regulatory domain with three phosphorylation sites (Ser380, Thr382 and Thr383) that regulate PTEN stability and may affect its biological activity [18]. In this study, we found that YSL significantly increased protein level of PTEN in BEL-7402 cell; meanwhile YSL also decreased inactivated PTEN (p-PTEN) in cancer cell (Figure 2). When PTEN acts on inositol phospholipids generated by the activation of the phosphoinositide 3-kinase (PI3K), AKT would be inhibited and CDK inhibitors p21 and p27 downstream are activated. Then cell cycle and cell proliferation of tumor cells would be under control [19]. Therefore, we determined the effect of YSL on the expression of p21 and p27 in BEL-7402 hepatocarcinoma cells. At all doses of YSL, expression of p27 and p21 protein was markedly induced (Figure 2). P53 is a key regulator of the cellular response to stress and is rapidly up-regulated in response to DNA-damaging agents [20]. Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis [21]. P53 levels are primarily controlled by the proto-oncprotein mouse double minute 2 (MDM2), which ubiquitinates p53 and facilitates its proteasomal degradation [22]. Inhibition of AKT by YSL decreased the phosphorylation level of MDM2 in hepatocarcinoma cell, and then increased the protein level of P53 which would accelerate death proceeding of tumor cells [21] (Figure 3).

The cell cycle consists of an initial growth phase (G1), DNA replication (S), a gap phase (G2) and mitosis (M), after which the cell may differentiate or enter the resting state (G0). At present, research suggests that there is an exceptional ability of proliferation and differentiation in tumor cells, and these tumor cells are deprived of the ability of cell cycle control [23]. After incubated together with YSL (0.8 and 1.6 mg/ml) for 48 h, the proportion of S-phase (proliferative phase) human hepatocellular carcinoma BEL-7402 cells was decreased, and the proportion of G0/G1 (DNA-synthesis resting phase) cells was increased (Figure 4).
Our previous study has shown that YSL destroys the mitochondrial structure and induces apoptosis in tumor cells [24]. As a serine-threonine kinase, AKT directly phosphorylates and inactivates proteins involved in apoptosis including BAD [25]. BAD is a death-promoting BH3-only member of the Bcl-2 family of proteins and its proapoptotic activity is inhibited by phosphorylation, in particular by AKT at Ser136 [26]. Phosphorylation is necessary for the association of BAD with 14-3-3 proteins that prevents BAD translocation to the mitochondria and interaction with Bcl-XL and Bcl-2 [27], thus allowing the latter proteins to promote cell survival. Therefore, the inhibition of AKT can result in potentiation of the mitochondrial pathway under appropriate apoptotic stimuli. Western Blot analysis showed that p-BAD (Ser136) decreased significantly with the inhibition of AKT by YSL treatment (p < 0.05). And the protein levels of Bcl-2 and Bcl-XL also decreased at the same time in hepatocarcinoma BEL-7402 cells (p < 0.05). Reduction of anti-apoptotic proteins Bcl-XL and Bcl-2 induces mitochondrial permeabilization and releases cytochrome c into cytoplasm, which triggers activation of the caspase cascade [26]. In all caspases, Caspase-3 is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP). Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments [28]. In this study, Cleaved Caspase3 in all YSL-treated groups was significantly higher than that in the control group (p < 0.05) (Figure 5).

Bcl2 and Bcl-xL prevents apoptosis through heterodimerization with an apoptotic protein inhibits its apoptotic effect [29] or formation of mitochondrial outer membrane pores help maintain a normal membrane state under stressful conditions [30]. The reduction of Bcl2 and Bcl-xL by YSL could cause instability of mitochondria membrane. Flow cytometric analysis further showed the decrease of mitochondria-labeled Rh123 fluorescence in YSL-treated groups,
Figure 5. Effect of YSL on protein expression of PTEN in human hepatocarcinoma BEL-7402. BEL-7402 cells were treated with YSL (0.8 mg/ml, 1.6 mg/ml) for 24 and 48 h. After drug administrations, total proteins of tumor cells were extracted. Western blot was performed with total protein to assay Phospho-Bad (Ser136), Bcl2, Bcl-XL and Cleaved Caspase 3 protein expression. Error line stands for SD, *: versus control group, $p < 0.05$, $N = 3$.

Figure 6. Effect of YSL on mitochondrial permeability transition pore of human hepatocarcinoma BEL-7402 in vitro. BEL-7402 cells were treated with YSL (0.8 mg/ml, 1.6 mg/ml) for 24 and 48 h. After drug administrations, mitochondrial permeability transition pore opening of tumor cells were measured using MitoProbe™ Transition Pore Assay Kit by FCM. The change in fluorescence intensity between Tube 1 (containing Calcein AM + CoCl₂) and Tube 2 containing (Calcein AM + CoCl₂ + Ionomycin) indicates the continuous activation of mitochondrial permeability transition pores. *: compared with control group, $p < 0.05$, $N = 6$. 

DOI: 10.3109/1061186X.2013.844820
which implied that YSL significantly disturbed the mitochondrial membrane potential of hepatocarcinoma cells. Since mitochondrial potential reduction is related to the mitochondrial membrane permeabilization, we then examined the effects of YSL on the mPTP opening. As expected, YSL treatment reduced the fluorescent signal of calcine on mitochondrion in BEL-7402 cells, suggesting that YSL promoted the mPTP opening (Figure 6).

Mitochondria may act as a spatial Ca$^{2+}$ buffer in many cells, regulating the local Ca$^{2+}$ concentration in cellular microdomains. The decrease in mitochondrial transmembrane potential and mPTP opening changed mitochondrial mem-

brane permeability and caused Ca$^{2+}$ to leak out from mitochondria to cytoplasm. Our result showed that the fluorescence of calcium indicator-Fluo 4 in BEL-7402 cells increased significantly after treated with YSL, suggesting calcium concentration in cytoplasm overloaded. Dysregulation of Ca$^{2+}$ homeostasis has long been implicated to play an important role in cell injury. Increased Ca$^{2+}$ activates a number of phosphatases, proteases and nuclease which may act on processes of signal transduction, gene expression in the cell and induce apoptosis [31] (Figure 7). To clarify whether YSL can indeed induce hepatocarcinoma cell apoptosis, we performed a series of apoptotic assays on BEL-7402 cell to elucidate the apoptosis promoting function of YSL. The results of the Annexin V assay, which examined the exposed phosphatidylserine (PS) on apoptotic cell membrane [32], showed that YSL could induce hepatocarcinoma cell BEL-7402 apoptosis in a dose-dependent manner (Figure 8). The DNA Ladder assay showed that in treated BEL-7402 cells, YSL promoted nuclear DNA fragmentation, which is detected as a "DNA ladder" on agarose gels and is a hallmark of apoptosis (Figure 9). Since oligo- and mononucleosomes are generated by internucleosomal cleavage of chromatin during apoptotic cell death, nucleosome ELISA allowed us to analysis the apoptosis promotion function of YSL in a quantitative way. Our results showed that oligo- and mononucleosomes in the YSL-treated group was significant higher than those in control group (Figure 10).

**Discussion**

Cell survival process is controlled by many complicated signal transduction pathways. In all these signal pathways, the PI3K/AKT pathway associated very close with mitochondria control apoptosis and calcium signal transduction. The PI3K/AKT pathway regulates multiple cellular processes, including cell proliferation, differentiation, survival, growth, motility and angiogenesis. PTEN and AKT is a couple of balancing factors with adverse function in this pathway. The AKT cascade is activated by the production of phosphatidylinositol 3,4,5 triphosphates (PtdIns(3,4,5)P3) by phosphoinositide 3-kinase (PI3K). Recent studies have identified the substrates of AKT that are involved in the pro-cell survival effects, which thus far include glycogen synthase kinase-3, mTOR, FKHR, MDM2, p21, p27, HIF-1, IKK, Bad and caspase-9.

PTEN is a phospholipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate and inhibits PI3K-dependent activation of AKT. The mutation or loss of PTEN leads to constitutively activated AKT. Over expression of PTEN into PTEN deficient breast, prostate, lung and glioblastoma cancer cells resulted in a decrease in activated AKT. Our results showed that YSL could up-regulate the mRNA and protein expression of tumor suppressor PTEN in tumor cells and increase their activities, meanwhile inhibited the mRNA and protein expression of oncogene AKT and decreased the activated kinase quantities of AKT. The resuming balance effect of YSL between PTEN and AKT could prevent the transmission of tumor cell proliferation signals in the PI3K/AKT pathway.

AKT regulates cell cycle and cell proliferation through its direct action on the CDK inhibitors p21Waf1/Cip1 and p27Kip1. They function in forming heterotrimeric complexes with cyclins and cyclin-dependent kinases. In association with CDK2 complexes, p21Waf1/Cip1 serves to inhibit kinase activity and block cell cycle progression through G1/S [33]. p21 Waf1/Cip1 can also inhibit the combination of proliferating-cell nuclear antigen and DNA polymerase, thus, inhibiting the extension of DNA. The ability of p27 to enforce the G1 restriction point is derived from its inhibitory binding to CDK2/cyclin E and other CDK/cyclin complexes [34]. Results from the current study indicate that YSL enhances the expression of both p21 and p27 by its inhibitory effect on activation of AKT. The increased expression of these proteins then results in cell cycle arrest and inhibition of tumor cell proliferation.

The other consequence of inhibitory effect on AKT by YSL is to decrease the AKT-mediated phosphorylation of MDM2. p-MDM2 binds the transcriptional activation domain of p53 and blocks its ability to regulate target genes, mediated ubiquitination and degradation of p53 and to exert anti-proliferative effects [35]. Reduction of p-MDM2 increased the p53 level in heptaocarcinoma BEL-7402, which exerts its anti-proliferative effects, including growth arrest and apoptosis [21].

AKT is a major mediator of cell survival through direct inhibition of pro-apoptotic signals such as Bad and the Forkhead family of transcription factors. Bad is a proapoptotic member of the Bcl-2 family that promotes cell death by displacing Bax from binding to Bcl-2 and Bcl-xL. BAD heterodimerizes with BCL-XL or BCL-2 through its BH3 domain, neutralizes their protective effect on stability of mitochondria membrane, increases the permeability of mitochondria membrane [26] and induces apoptosis. AKT phosphorylates Bad at Ser136, blocking the dimerization of Bad and the anti-apoptotic proteins Bcl-xL or Bcl-2. Inhibitory effect of YSL on AKT reduces its expression [34]. Results from the current study indicate that YSL increases the expression of both p21 and p27 by its inhibitory effect on activation of AKT. The increased expression of these proteins then results in cell cycle arrest and inhibition of tumor cell proliferation.
mitochondria is the enhancement of calcium in cytoplasm. As one site of calcium storage, the mitochondria are very important in buffering calcium overload in cells. Permeabilization of the mitochondrial membrane induced by YSL caused the calcium leak into cytoplasm. Increased Ca\textsuperscript{2+} activates a number of phosphatases, proteases and nucleases, which may act on processes of signal transduction, gene expression in the cell and induce apoptosis.

Figure 7. Change of cytoplasmic-free calcium in BEL-7402 treated by YSL \textit{in vitro}. BEL-7402 cells were treated with YSL (0.8, 1.6 mg/ml) for 24 and 48 h. After drug administration, cancer cells were loaded with calcium indicator Flou-4 AM, and fluorescence was measured using Fluoroscan Ascent microplate fluorometer. *: compared to control group, \(p < 0.05\), \(N = 5\). A: control, 24 h; B: YSL 0.8 mg/ml, 24 h; C: YSL 1.6 mg/ml, 24 h; D: control, 48 h; E: YSL 0.8 mg/ml, 48 h; F: YSL 1.6 mg/ml, 48 h.
In conclusion, the mechanism of anticancer effect of YSL might be its resuming balance function between PTEN and AKT, which prevents the transmission of tumor cell proliferation signals in the PI3K/AKT pathway. Inhibition of AKT would change the status of downstream effectors in the PI3K/AKT pathway: (1) Inhibition of AKT up-regulated expression of cell cycle regulatory factors of downstream – P21 and P27 which repressed cell cycle and inhibited proliferation of tumor cells.

Figure 8. Effect of YSL on apoptosis of human hepatocarcinoma BEL-7402 by Annexin V assay (24 h). BEL-7402 cells were treated with YSL (0.8, 1.6 mg/ml) for 24 h. After drug administration, apoptosis of tumor cells were measured by Annexin V assay using flow cytometry. A: Control; B: YSL 0.8 mg/ml; F: YSL 1.6 mg/ml. *: compared with control group, $p < 0.05$, $N = 6$.

Figure 9. Apoptotic DNA fragments induced by YSL in human hepatocarcinoma BEL-7402 cells. BEL-7402 cells were treated with YSL (0.8, 1.6 mg/ml) for 48 h. Total DNA from tumors was prepared, and fragmentation was assessed by 2% agarose gel electrophoresis. Lane 1: YSL 0.8 mg/ml; Lane 2: 100 bp DNA Marker; Lane 3: Control; Lane 4: YSL 1.6 mg/ml.

Figure 10. Effect of YSL on production of apoptosis free nucleosomes DNA in human hepatocarcinoma BEL-7402. BEL-7402 cells were treated with YSL (0.8, 1.6 mg/ml) for 48 h. Nucleosome ELISA Kit was used to measure the quantity of apoptosis free nucleosomes DNA. Quantitation is achieved by the construction of a standard curve using standards with designated nucleosome unit values. Error line stands for SD, *: versus saline control group, $p < 0.05$, $N = 3$.

Conclusions

In conclusion, the mechanism of anticancer effect of YSL might be its resuming balance function between PTEN and AKT, which prevents the transmission of tumor cell proliferation signals in the PI3K/AKT pathway. Inhibition of AKT would change the status of downstream effectors in the PI3K/AKT pathway: (1) Inhibition of AKT up-regulated expression of cell cycle regulatory factors of downstream – P21 and P27 which repressed cell cycle and inhibited proliferation of tumor cells.
cells. (2) Inhibition of AKT decreased the phosphorylation level of MDM2, and then increased the protein level of P53, which would accelerate death proceeding of tumor cells. (3) Inactivation of AKT removed its inhibition effect on phosphorylation of Bad, which might decrease protein level of apoptosis inhibitor Bcl-2 and Bcl-XL, damaging mitochondria of tumor cells and inducing apoptosis.

Declaration of interest
The authors report no declarations of interest.

This work was supported by Grant sponsor: National Basic Research Program (973 Program, China) (Grant number: 2009CB918903); and Natural Science Foundation of Tianjin (Grant number: 09JCZDJC19700); Ph.D. Programs Foundation of Ministry of Education of China (Grant number: 20101202110001); and Cooperation Project in industry, education and research of Guangdong province (Grant number: 2009B090300430).

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