Vincosamide inhibits malignant behaviors of hepatocellular carcinoma cells by activating caspase-3 activity and blocking the PI3K/AKT signaling pathway

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Abstract

**Background:** Vincosamide (Vinco) was first identified in the methanolic extract of the leaves of *Psychotria leiocarpa*, and Vinco has important anti-inflammatory effects and activity against cholinesterase, Vinco also has a trait to ant-tumor. However, whether Vinco is able to inhibit the malignant behaviors of hepatocellular carcinoma (HCC) cells is still unclear. In the present study, we explored the role of Vinco in suppressing the malignant behaviors of HCC cells.

**Methods:** MTT, trypan blue exclusion assay and the Cell Counting Kit (CCK)-8 analysis were applied to detect the proliferation and death of HCC cells; electron microscopy was performed to observe the change of cellular mitochondrial morphology; scratch repair and Transwell assays were used to analyze the migration and invasion of HCC cells; the expression and localization of proteins were detected by laser confocal microscopy and Western blotting; and the growth of the cancer cells in *in vivo* was assessed in a mouse tumorous model.

**Results:** At a dose of 10-80 mg/ml, Vinco inhibited the proliferation and promoted the death of HCC cells in a dose-independent manner, but had low cytotoxicity effect on normal liver cells. Additionally, 80 mg/ml of Vinco could significantly disrupt the morphology of mitochondria, suppress the migration and invasion of HCC cells. The growth of HCC cells in the animal tumorous model was significantly inhibited after treatment with Vinco (10 mg/kg/day) for 3 days. The results of the present study indicated that Vinco (10-80 mg/ml) played a role in activating caspase-3, promoting the expression of PTEN, and inhibiting the phosphorylation of AKT (Ser473) and mTOR (Thr2448), Vinco also has a trait for suppressing the expression of CXCR4, Src, MMP9, EpCAM, Ras and Oct4 in HCC cells.

**Conclusions:** Vinco has a role in inhibiting the malignant behaviors of HCC cells; the role molecular mechanism of Vinco maybe involve in restraining expression of the growth-, metastasis-related factors Src, Ras, MMP9, EpCAM and CXCR4, and activating the activity of caspase-3. Vinco also could block PI3K/AKT signaling pathway. Thus, Vinco is an available chemotherapy for HCC patients.

Introduction

The genus *Psychotria* (Rubiaceae) contains more than 2000 species that mostly grow in tropical and subtropical regions [Marques de Oliveira et al., 2013]. Vincosamide (Vinco) was first identified in the methanolic extract from the leaves of *Psychotria leiocarpa*. *Palicourea* species (including *Psychotria leiocarpa*) have been used in folk medicine for the treatment of some disorders, including inflammatory disorders and cancer [de Moura et al., 2020; Volobuff et al., 2019; Formagio et al., 2019]. Previously, documents showed that Alkaloid extracts of *Psychotria Forsteriana* (include Vinco) has a cytotoxicity on rat hepatoma cell Lines [Adjibadé et al., 1989]. Some evidences indicated that Vinco was able to inhibit the growth of glioma cell lines [de Moura et al., 2020], protect against inflammatory diseases and respiratory disturbances, and exert anti-hallucinogenic effects [Caballero-George et al., 2001]. Monoterpene indolic alkaloid (N-β-glucopyranosyl vincosamide) is the main component of extracts from...
the leaves of *Palicourea* species collected from Morro Santana in Porto Alegre, Brazil [Matsuura et al., 2013]. It has been reported that Vinco has pharmacological effects, such as anti-oxidant effects and anti-mycobacterial effects, for example, against the growth of *Mycobacterium bovis* [Moraes et al., 2011], and has an analgesic activity [McKenna et al., 1984]. Additionally, it exhibits anti-inflammatory effects and dose-independent analgesic activity, and its effects are not reversible by naloxone. Vinco also plays a role in antagonizing the myocardial cells toxicity that induced by doxorubicin [Cheraghi et al., 2017], and inhibiting the growth of glioma cell lines [de Moura et al., 2020] and hepatoma cells [Adjibadé et al., 1989]. These findings suggested that Vinco not only has anti-inflammatory and anti-oxidant effects and inhibits the growth of cancer cells, but also has low cytotoxicity in normal cells. Although Vinco has been reported to possess these pharmacological effect, the role of Vinco in hepatocellular carcinoma (HCC) cells is still unclear, and the bio-target of Vinco in suppressing the malignant behaviors of cancer cells has not been determined.

Considering that species of the genus *Psychotria* have been used as anti-inflammatory agents, anti-microbial drugs and cancer treatments, but potential therapeutic applications of Vinco is still lack a scientific evidence, So research on the potential therapeutic effect of Vinco was performed in this study. The goal of this study was to explore the effect of Vinco on the malignant behaviors of HCC cells *in vitro* and its anti-cancer role in a mouse model *in vivo*.

**Material And Methods**

**Cell culture**

The human normal liver cell line L-02 and the human HCC cell lines HLE, Bel 7402 and PLC/PRF/5 were selected for the present study. These cells were gifts from the Department of Cell Biology and Department of Biochemistry and Molecular Biology, Peking University Health Science Centre (Beijing, China). The cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) and were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ as previously described [Li et al., 2002; Zhu et al., 2017].

**MTT assay**

L-02, HLE, Bel 7402 and PLC/PRF/5 cells were digested with trypsin and diluted in RPMI 1640 medium containing 10% FCS to form a suspension of $2.5 \times 10^4$ cells/ml, and 200 µl/well was subcultured in 96-well plates. After incubation for 48 h in the plates, the cells were treated with different concentrations of Vinco (5, 10, 20, 40, or 80 µg/ml) (purchased from Chengdu Greenpurify Biology Medicine Company, Chengdu, China. Purity 99.7%) for 48 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) solution (5 mg/ml) was added to the cells in each well, and the cells were cultured for another 4 h. The culture medium containing MTT was discarded, and 200 µL of dimethylsulfoxide was added to each well. The plates were oscillated for 10 min. The absorbance values of the experimental group were measured by a microplate reader (Bio-Rad) at a wavelength of 490 nm. The growth ratio was
calculated by the following formula: growth ratio = (treated $A_{490}$ /control $A_{490}$) × 100%. The procedure and calculations were based on a previous description [Li et al., 2002].

**CCK-8 assay**

The Cell Counting Kit (CCK)-8 (CK04, Dojindo, Tokyo, Honshu, Japan) was used to assess the effect of Vinco on the cell proliferation. L-02, HLE, Bel 7402 and PLC/PRF/5 cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere of 5% CO$_2$ for 48 h, and the cells were seeded at a density of $2.5 \times 10^4$ cells/well in a 96-well microplate. Then the cells were treated with Vinco (10 µg/ml and 80 µg/ml) for 24 h, 48 h and 72 h respectively, and a total of 10 µl of CCK-8 solution was added to each well. The detected operation according to the procedure of the Kit. The absorbance at 450 nm was measured using a Universal Microplate Reader (ELx800). The percentage of cell proliferation (viability) was calculated as the following formula: Proliferation ratio = (untreated $A_{450}$ - treated $A_{450}$) / (untreated $A_{450}$) × 100%.

**Trypan blue exclusion dye method to analyze cell viability and metabolic activity**

To evaluate cell viability, L-02, HLE, Bel 7402 and PLC/PRF/5 cells were seeded at a density of $2.5 \times 10^4$ cells/well in 6-well plates, and the cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere of 5% CO$_2$ for 48 h. Following treatment with different concentrations of Vinco (10 µg/ml or 80 µg/ml) for 48 h, cell viability was determined by the trypan blue exclusion dye assay using a Trypan Blue Staining Cell Viability Assay Kit (Beyotime Biotech Corp, Haimen, Jiangshu, China). Cells restricted trypan blue entry were considered viable. Five different visual fields were used to analyze the quantity of cell viability ratio, the cell viability ratio was calculated according to the following formula: (total cells - Trypan blue stained cells) / total cells × 100%. The procedure and calculations were based on as previously described [Feng et al., 2019].

**Microscopy and DAPI nuclear staining to observe cell morphology**

To observe the alterations of cellular morphology that induced by Vinco, L-02, HLE, Bel 7402 and PLC/PRF/5 cells were plated at a density of $2.0 \times 10^4$ /ml in 24-well plates. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere of 5% CO$_2$ for 48 h, and then treated with 10 µg/ml or 80 µg/ml Vinco. After treatment for 48 h, cellular morphology was observed under a light microscope, and the cells were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution. The cells were imaged using a fluorescence microscope at 100 × magnification. In this study, nuclear pyknosis and fragmentation were used as evidence of apoptosis, and these criteria were evaluated by fluorescence microscopy as previously described [Zhu et al., 2016a; Zhu et al., 2016b], and five different visual fields were used to analyze the quantity of nuclear pyknosis, the formula: nuclear pyknosis (%) = numbers of nuclear pyknosis / total nucleus × 100%.

**Electron Microscopy To Observe Mitochondria**
HLE, Bel 7402 and PLC/PRF/5 cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere of 5% CO₂. The cells were treated with Vinco(80 µg/ml) for 48 h and then harvested, washed with phosphate-buffered saline (PBS) two times (4 °C, 10 ml, 8 min), and transferred to 1.5-ml EP tubes. The cells were fixed, and monolayer section of cells were prepared. The ultrathin sections were examined under a TECNA 10 transmission electron microscope (Philips, Holland). The morphological characteristics of cellular mitochondria were observed by randomly selecting ten cells from each group. Mitochondrial damage was evaluated by electron microscopy as previously described [Wang et al., 2019].

**Cellular Wound Repair Assay**

Cell motility was analyzed by a wound repair assay. HLE, Bel 7402 and PLC/PRF/5 cells (5 × 10⁴ cells/ml) were seeded in 12-well plates and allowed to reach almost total confluence in 24 h. A scratch was made by scraping the middle of the cell monolayer with a sterile micropipette tip. The cells were cultured with RPMI 1640 medium containing 10% FCS and Vinco(10 µg/ml). Then, images of the cells that migrated into the wound area were captured at 0, 24, and 48 h by an inverted microscope (100×), and their distances traveled were recorded. Cell repair motility was evaluated using the following formula: cell repair ratio (%) = (distance at 0 h-distance at X h)/distance at 0 h × 100%, with X representing the time points of observation. The procedure was performed as previously described [Feng et al., 2019].

**Cell Invasion Assay**

The cell invasion assay was carried out on HLE, Bel 7402 and PLC/PRF/5 cells according to the manufacturer's protocols. Cell invasion was measured by observing cells cultured on inserts in Transwell chambers (Transwell chamber; 8-mm pore size; Costar, High Wycombe, UK) covered with Matrigel (BD Falcon, NJ, USA). The cells were plated in 12-well culture plates with separate upper and lower chambers. The cells were added to the upper chambers (5 × 10⁴ cells/ml), cultured with serum-free RPMI 1640 medium and treated with Vinco(10 µg/ml and 80 µg/ml), whereas the lower chamber was filled with complete medium (containing 20% FCS). After 48 h of incubation, the cells in the upper chamber were carefully removed with a cotton swab, and those that had invaded through the membrane to the lower surface were fixed with 90% methanol and stained with 0.1% crystal violet. The number of cells that had invaded through the membrane was quantified by counting the cells in five different visual fields under a microscope (Olympus) with a 20 × objective. Three independent assays were performed.

**Laser Confocal Microscopy**

HLE, Bel 7402 and PLC/PRF/5 cells were stained as described previously [Li et al., 2013]. To observe the migration of caspase-3 molecules, cells were treated with Vinco(10 µg/ml and 80 µg/ml); to evaluate the expression and location of Src, Oct4 and PTEN, cells were treated with Vinco(80 µg/ml). Laser confocal microscopy was performed to observe the expression and location of the target proteins. Briefly, cells were fixed in 4% paraformaldehyde and incubated with mouse anti-human caspase-3, anti-Src antibodies, rabbit anti-human Oct4, anti-PTEN antibodies (Abcam Trading (Shanghai) Company, Ltd., Shanghai,
China) for 24 h. Alex 488-, Alex 647-, or fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse immunoglobulin G (IgG) was added, and the cells were incubated for 2 h. Afterwards, 100 µl of DAPI (1 µg/ml) was added for 30 min. The cells were visualized with a Leica TCS-NT SP2 laser confocal microscope (Leica Camera, Wetzlar, Germany).

**Western Blotting Analysis**

To estimate the influence of Vinco on the expression of proteins related to metastasis and apoptosis, and the PI3K/AKT signaling pathway, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco (10 µg/ml or 80 µg/ml) for 24 h. The expression of metastasis-related proteins, such as MMP9, CXCR4, and EpCAM; apoptosis-related proteins, such as activated caspase-3, PARP-1, PTEN, Bax, and Bcl-2; and factors regulated by the PI3K/AKT signaling pathway, such as pAKT (Ser473), and pmTOR (Thr2448) in these cells was analyzed by Western blotting as previously described [Li et al., 2013].

**Immunohistochemical Analysis**

The protein expression of c-myc, Ras, activated caspase-3 and PARP-1 in tumor-bearing mice were evaluated by immunohistochemical analysis. Briefly, tumorous tissue was removed from tumor-bearing mice and cut into five-millimeter-thick paraffin sections. The sections were deparaffinized and rehydrated according to standard protocols. The sections were then incubated with primary antibody (1:100 dilution; Abcam Trading Company, Ltd. Shanghai, China) at 4 °C overnight. Nonimmune (IgG) was used as a negative control, and antigenic sites were identified using an SP9000 Polymer Detection System and a 3,3′-diaminobenzidine Kit (ZSGB-BIO, Beijing, China). The standard protocols were performed in accordance with approved guidelines [Li et al., 2013; Lu et al., 2016].

**Analysis Of Caspase3 Activity**

HLE, Bel 7402 and PLC/PRF/5 cells were treated with tumor necrosis factor-related apoptosis-induced ligand (TRAIL) (2 µmol/L) or Vinco (80 µg/ml) and a caspase-3 inhibitor (Z-DEVD-FMK, 1.0 µmol/L) (Selleck Chemicals Company, USA) for 24 h. Caspase-3 activity was measured with a commercial Kit (APOP-CYTO Caspase-3 Colorimetric Assay Kit; Medical and Biological Laboratories, Japan) according to the manufacturer’s protocol as described in a previous study [Li et al., 2009].

**Animal Experiments**

Male pathogen-free athymic nude mice (BALB/C, male, 6 weeks, 20–25 g) were purchased from the Guangzhou Animal Research Center (Guangzhou, China), the mic were fed in no specific pathogen environment, and sterile feed. The animals were maintained in a facility approved by the Ethical Committee of Hainan Medical University. The experimental procedures were approved by the Hainan Medical College Institutional Committee. To analyze tumorigenicity, Bel 7402 and PLC/PRF/5 cells (1 × 10^6) in 0.1 ml of Hank's balanced salt solution were subcutaneously injected into the right scapular region of nude mice (10 per group). The mice were intraperitoneally injected with Vinco (10 mg/kg/day)
every day. Tumor-bearing mice were killed every 3 days after inoculation (during the fed, the lethality rate is 30%), tumorous tissues were removed, the length (L) and weight (W) of the tumors were measured, and the volume (V) was calculated by the following formula as described previously[Euhus et al., 1986; Tomayko et al., 1989], \( V = \frac{\pi}{6} \times L \times W^2 \) (\( \pi = 3.14 \)). On day 21, tumorous tissues, and the expression of activated caspase-3, PARP-1, PTEN and pAKT(Ser473) was detected by immunohistochemical analysis or Western blotting.

**Statistical analysis**

The data are presented as the mean ± S.D. Statistical analysis was performed using Student’s t-test (for two experimental groups). Significance was set at \( P< 0.05 \). Statistical significance was determined using Student’s t-test, and Kruskal-Wallis test the F test (SPSS 11.5 software for Windows, SPSS Inc., Chicago, IL, US).

**Results**

**Vinco inhibited the proliferation of HCC cells and promoted their apoptosis**

First, display the molecular structure of Vinco in Fig. 1A. In the MTT assay, L-02, HLE, Bel 7402 and PLC/PRF/5 cells were treated with different concentrations of Vinco(5–80 µg/ml) for 48 h. At a concentration of 10 µg/ml of Vinco inhibited the growth of HLE cells, but had weak effect on the growth of Bel 7402 and PLC/PRF/5 cells. When the concentration of Vinco was greater than 40 µg/ml, the proliferation of HLE, Bel 7402 and PLC/PRF/5 cells was significantly suppressed; the results also revealed that Vinco had little effect on normal human liver L-02 cells (Fig. 1B). CCK-8 detected results also indicated that concentration of 10 µg/ml of Vinco was able to inhibited the proliferation of HLE cells while treated for 48 h and 74 h, but weak effect on the proliferation of L-02, Bel7402 and PLC/PFR/5 cells(Fig. 1C); and the high concentration of Vinco(80 µg/ml) significantly suppressed the proliferation of L-02, HLE, Bel7402 and PLC/PFR/5 cells while treated for 24 h, 48 h and 72 h, but the effect more higher in HLE, Bel7402 and PLC/PFR/5 cells than in L-02 cell (Fig. 1D). To further evaluate the role of Vinco in viability and apoptosis of HCC cells, L-02, HLE, Bel 7402 and PLC/PRF/5 cells were treated with different concentrations of Vinco(10 µg/ml or 80 µg/ml) for 48 h. We applied trypan blue exclusion dye method to analyze cell viability and metabolic activity, and observed the changes in cellular morphology by microscopy. The results showed that the number of dead cells in the Vinco-treated groups were significantly increased that compared to the untreated groups(Fig. 1E), and cell morphological changes occurred in HLE, Bel 7402 and PLC/PRF/5 cells while treated with Vinco(Fig. 1F). DAPI staining results showed that nuclear morphological changes were observed in HLE, Bel 7402 and PLC/PRF/5 cells(Fig. 1G), the degree of cellular nuclear condensation and pyknosis was significantly increased compared to that in untreated cells, and morphological characteristics of apoptosis, including nuclear shrinkage, were apparent. However, few changes were observed in the untreated group and in normal liver cell(L-02) (Fig. 1G). These results indicate that Vinco played a role in inhibiting the growth, metabolism and promoting death of HCC cells.
Vinco disrupted the morphology and function of mitochondria in HCC cells

To reveal the role of Vinco in suppressing the growth of HCC cells and stimulating their apoptosis, we used electron microscopy to observe morphological and structural changes of mitochondria in HCC cells. HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(80 µg/ml) for 48 h. The results indicated that mitochondrial swelling and ridge breakage occurred in apoptotic HCC cells, but the morphology of mitochondria in the untreated groups remained normal (Fig. 2). These results demonstrated that Vinco was able to disrupt the morphology and function of mitochondria in HCC cells.

Vinco inhibited the migration and invasion of HCC cells

To observe the effect of Vinco on the migration and invasion of HCC cells, cellular wound repair and invasion assays were performed. In the cellular wound repair assay, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(10 µg/ml) for 24 h or 48 h. The scratch repair assay results revealed that repair migration of Vinco-treated HLE cells was significantly decreased compared to that of untreated HLE cells. Few change was observed in Bel 7402 and PLC/PRF/5 cells while treated with Vinco(10 µg/ml) for 24 h, but when these cells were treated with Vinco(10 µg/ml) for 48 h, their repair migration was significantly decreased compared to that of untreated (control) cells (Fig. 3A). The invasion assay indicated that the pore transfer capacity of HLE, Bel 7402 and PLC/PRF/5 cells which were treated with Vinco(10 µg/ml or 80 µg/ml) for 48 h were significantly decreased compared to that of untreated cells (control); the results also showed that the pore transfer capacity of HCC cells which were treated with a high concentration of Vinco(80 µg/ml), was significantly decreased compared to that of HCC cells which were treated with a low concentration of Vinco(10 µg/ml) (Fig. 3B). These results reveal that Vinco could inhibit the migration and invasion of HCC cells in a dose-dependent manner.

Furthermore, we performed Western blotting to explore the effect of Vinco on the expression of migration- and invasion-related proteins. The results indicated that the expression of MMP9, CXCR4 and EpCAM was significantly reduced in HLE cells while treated with Vinco(10 µg/ml or 80 µg/ml) for 48 h, compared to that in untreated cells (control) (Fig. 3C). The expression of these proteins was also significantly lower in HLE cells while treated with a high concentration of Vinco (80 µg/ml) than in those treated with a low concentration of Vinco(10 µg/ml). Bel 7402 cells were treated with Vinco(10 µg/ml or 80 µg/ml) for 48 h, the expression of MMP9 and CXCR4 was significantly reduced compared to untreated cells (control) (Fig. 3C), and the expression of these proteins was also significantly lower in Bel 7402 cells while treated with a high concentration of Vinco(80 µg/ml) than that were treated with a low concentration of Vinco(10 µg/ml). However, there was weak change in the expression of EpCAM in Bel 7402 cells. PLC/PRF/5 cells were treated with Vinco(10 µg/ml or 80 µg/ml) for 48 h, the expression of CXCR4 and EpCAM were significantly reduced compared to untreated cells (control) (Fig. 3C), and the expression of these proteins were also significantly lower in PLC/PRF/5 cells while treated with a high concentration of Vinco(80 µg/ml) than that were treated with a low concentration of Vinco(10 µg/ml). However, there was few change in the expression of MMP9 in PLC/PRF/5 cells (Fig. 3C). These results demonstrated that Vinco has a function for inhibiting the expression of migration- and invasion-related proteins.
Vinco activated caspase-3 activity in HCC cells

Activated caspase-3 activity is a pivotal factor for promoting apoptosis of cell. In the present investigation, we applied laser confocal microscopy to observe the expression, location and migration of caspase-3. The results showed that HLE, Bel 7402 and PLC/PRF/5 cells expressed caspase-3 (green fluorescence) and that the caspase-3 protein was located in the cytoplasm in these cells (Fig. 4A). The results also showed that a small amount of caspase-3 molecules migrated from the cytoplasm to nucleus when the cells were treated with a low concentration of Vinco (10 µg/ml) for 48 h. However, a large amount of caspase-3 molecules were assembled in the nucleus (green fluorescence) when the cells were treated with a high concentration of Vinco (80 µg/ml) for 48 h (Fig. 4A). To explore the effect of Vinco on the expression of apoptosis-related proteins, Western blotting was used to detect the expression of Bax, Bcl-2, activated caspase-3 and PARP-1. The results indicated that the expression of Bax was significantly stimulated, but the expression of Bcl-2 was significantly inhibited, and the expression of activated caspase-3 and PARP-1 was significantly promoted in HLE, Bel 7402 and PLC/PRF/5 cells while treated with Vinco (10 µg/ml or 80 µg/ml) for 48 h (Fig. 4B). The Bax/Bcl-2 ratio was also elevated in these cells while treated with Vinco (10 µg/ml or 80 µg/ml), but the elevation in the ratio was more significant in the cells while treated with a high concentration of Vinco (80 µg/ml) than that treated with a low concentration of Vinco (10 µg/ml), the elevation of Bax/Bcl-2 ratio imply that promotion of apoptosis (Fig. 4C). Furthermore, we analyzed the effect of Vinco on the activity of caspase-3. HLE, Bel 7402 and PLC/PRF/5 cells were treated with tumor necrosis factor-related apoptosis-induced ligand (TRAIL), which promotes TRAIL receptor-mediated caspase-3 activity, as a positive control, and Z-DEVD-FMK, which inhibits caspase-3 activity for 48 h. The results indicated that Vinco (80 µg/ml) was able to activate caspase-3 activity similarly to TRAIL (2 µmol/L), and the inhibitor (Z-DEVD-FMK) blocked the effect of Vinco. Vinco synergized with TRAIL to stimulate caspase-3 activity (Fig. 4D). These results proved that Vinco has the capacity to activate the activity of caspase-3 in HCC cells.

Vinco blocked PI3K/AKT signaling pathway and inhibited the expression of oncogenes

In the present study, we explored the effect of Vinco on the PI3K/AKT signaling pathway and the expression of oncogenes. HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco (80 µg/ml) for 48 h, and the expression and location of PTEN, Src and Oct4 were observed by laser confocal microscopy. The results indicated that Vinco promoted the expression of PTEN and that PTEN was located in the cytoplasm (Fig. 5A). The expression of Src and Oct4 was significantly inhibited, Src was located in the cytoplasm and Oct4 was located in the nucleus (Fig. 6A and B). We also used Western blotting method to detect the expression of PTEN and other proteins which related to the PI3K/AKT signaling pathway. The results revealed that the expression of PTEN was significantly stimulated and that the expression of pAKT (Ser473), pmTOR (Thr2448) was significantly inhibited when these cells were treated with Vinco (10 µg/ml or 80 µg/ml) for 48 h (Fig. 5B). Additionally, a high concentration of Vinco (80 µg/ml) has a greater effect on suppressing the expression of pAKT (Ser473) and pmTOR (Thr2448) in HCC cells (Fig. 5B). Vinco not only inhibited the expression of pAKT (Ser473), Src and Oct4, but also synergized with a PI3K inhibitor (Ly294002) to inhibit the expression of pAKT (Ser473), Src and Oct4 (Fig. 6C). These results
proved that Vinco was able to block the activation of the PI3K/AKT signaling pathway and inhibit the expression of oncogenes \textit{in vitro}.

**Vinco inhibited cancer growth, activated caspase-3 activity and blocked the PI3K/AKT signaling pathway in vivo**

In the present study, the therapeutic effect of Vinco on a tumorous model was observed by injecting Bel 7402 and PLC/PRF/5 cells into the right scapular region of male nude mice. The mice were intraperitoneally injected with Vinco (10 mg/kg/day) every day, tumor-bearing mice were killed every 3 days after inoculation, and the tumorous volume was calculated. The results showed that the tumorous volume of Vinco-treated mice was significantly reduced compared to that of untreated mice (control) at the same time point after 9 days (Fig. 7A and B). Furthermore, immunohistochemical analysis was applied to detect the expression of c-myc, Ras, activated caspase-3 and PARP-1 in tumorous tissues at 21 days. The results indicated that the expression of c-myc and Ras was significantly decreased in tissues which extracted from Vinco-treated mice compared to the tissues which extracted from untreated mice, but the expression of activated caspase-3 and PARP-1 was significantly elevated in tissues which extracted from Vinco-treated mice compared to the tissues which extracted from untreated mice (Fig. 7C and D). Western blotting results revealed that the expression of activated caspase-3, PARP-1 and PTEN was significantly elevated in tissues which extracted from Vinco-treated mice compared to the tissues which extracted from untreated mice, but the expression of pAKT (Ser473) was significantly decreased (Fig. 7E). These results further demonstrated that Vinco inhibited the growth of liver cancer cells, activated caspase-3 activity and blocks transduction of PI3K/AKT signaling pathway.

**Discussion**

Monoterpene indolic alkaloid (N-\(\beta\)-glucopyranosyl vincosamide) is the main component of extracts from the leaves of \textit{Psychotria leiocarpa}, which is found in tropical and subtropical regions. Many studies have shown that Vinco has anti-inflammatory activities \textit{in vivo} and anti-proliferative effects \textit{in vitro}[Marques de Oliveira et al 2013; Matsuura et al., 2013; Benevides et al., 2004; Li et al., 2017], and Vinco also has cytotoxic and anti-bactericidal activities[de Moura et al., 2020; Mahmud et al., 1993]. Some investigations over the past three decades have indicated that Vinco inhibited the proliferation of cancer cells and promotes their apoptosis[Volobuff et al., 2019; Adjibadé et al., 1989], and that it antagonized cardiotoxicity that induced by doxorubicin in rats \textit{in vitro} and \textit{in vivo}[Cheraghi et al., 2017]. These evidences indicated that Vinco played a complex role in regulating anti-inflammatory and anti-bacterial activities and antagonizing cardiotoxicity, it also has a capacity to inhibit the growth of cancer cells.

Although evidence has revealed that an analog of Vinco induced cytotoxicity in human leukemic and rat hepatoma cell lines[Adjibadé et al., 1989], inhibited the growth of a glioma cell line (U251)[de Moura et al., 2020] and has anti-oxidant activity to protect against oxidative stress generated upon wounding[Matsuura et al., 2013]; the effect of Vinco on the malignant behaviors of human HCC cells is still unclear. In the present study, we observed the cytotoxicity of Vinco in human HCC cells and human
normal liver cells. The results of the MTT, CCK-8 and trypan blue exclusion assays demonstrated that Vinco significantly inhibited the growth of HCC cells and promoted their death at concentrations greater than 40 µg/ml but had weak cytotoxicity in normal liver cells. DAPI staining also showed that Vinco promoted cellular nuclear condensation and pyknosis, these are the classic feature of apoptosome. Additionally, the cellular wound repair and invasion assays indicated that Vinco significantly suppressed the migration and invasion of HCC cells. To our surprise, the low concentration of Vinco (10 µg/ml) inhibited the growth of HLE cells, implying that the sensitivity of different HCC cells to Vinco is diverse, possibly due to their heterogeneity. Previously, we have found that HLE cells are more sensitizite to other agents than else HCC cells, such as benzyl isothiocyanate and paclitaxel, due to the lower expression of alpha fetoprotein (AFP) in HLE cells compared to that in Bel 7402 and PLC/PRF/5 cells, and that AFP has an antagonistic effect on the ability of these drugs to induce apoptosis of HCC cells[Zhu et al., 2016a; Zhu et al., 2016c]. AFP has a trait to inhibit autophagy and immune response, led to the occurrence and initiation of liver cancer[Wang et al., 2018; Zhu et al., 2020; Zheng et al., 2020; Li et al., 2020], this is a crucial factor for the drug-resistance of HCC cells. However, these results also demonstrate that Vinco has the capacity to inhibit growth and invasion, and stimulate apoptosis of HCC cells.

To explore the mechanism underlying the ability of Vinco to suppress the malignant behaviors of HCC cells, we evaluated the changes in the morphology and function of cellular mitochondria. The results indicated that Vinco significantly disrupted mitochondrial morphology. Western blotting analysis displayed that the expression of Bcl-2 was significantly suppressed, but the expression of Bax, cleaved caspase-3, and PARP-1 were significantly stimulated, and the activity of caspase-3 was activated. Vinco also promoted the migration of activated-caspase-3 molecules from the cytoplasm to the nucleus. These results suggested that Vinco was able to impair the function of mitochondria in HCC cells and activate the activity of caspase-3 in vitro. The activated PI3K/AKT signaling pathway is a pivotal factor for promoting the proliferation, drug resistance, and metastasis of HCC cells[Tu et al., 2016; Fu et al., 2018; Li et al., 2013]. Recently, many studies have indicated that activated PI3K/AKT signaling pathway was able to stimulate the expression of malignant behavior-related genes, such as Src, CXCR4, MMP2/9, and EpCAM[Xia et al., 2013; O’Brien et al., 2010; Zhu et al., 2015; Tian et al., 2010]. PTEN is critical molecule for inhibiting the activation of PI3K/AKT signaling pathway in HCC cells[Wang et al., 2018; Chen et al., 2014; Zhu et al., 2020; Fu et al., 2017]. The results of this study indicate that Vinco inhibits the expression of Src, CXCR4, MMP9 and EpCAM, and stimulates the expression of PTEN, leading to the expression of pAKT(Ser473) and pmTOR(Thr2448) were inhibited. These results reveal that Vinco inhibited transduction of PI3K/AKT signaling pathway is critical for suppressing the malignant behaviors of HCC cells.

Vinco has the capacity to inhibit the proliferation of HCC cells in vitro, but whether it has the same effect in vivo is still unclear. In the present study, we observed the therapeutic effect of Vinco on an animal tumorous model. Animal tumorous models were administered Vinco(10 mg/kg/day) every day. Because HLE cells can not induce production of tumors when transplanted into mice, only Bel 7402 and PLC/PRF cells were selected to inject into the mice. The results showed that the tumorous volume of Vinco-treated mice was significantly reduced compared to untreated mice. The results also showed that the expression
of c-myc, Ras, and pAKT(Ser473) significantly decreased in tissues which extracted from Vinco-treated mice compared to the tissues which extracted from untreated mice, but the expression of activated caspase-3, PARP-1 and PTEN was significantly elevated. These results further to prove that Vinco suppressed the growth of liver cancer cells, and it was able to activate the activity of caspase-3, and stimulated the expression of PTEN to block the transduction of PI3K/AKT signaling pathway in vivo. We have summarized the mechanism underlying the role of Vinco in inhibiting the malignant behaviors of HCC cells(Fig. 8).

Conclusions

The present study reported for the first time that Vinco has a effect on inhibiting the malignant behaviors of HCC cells. The results imply that caspase-3 and PTEN molecules maybe are the pivotal target for the role of Vinco. Overall, the role mechanism of Vinco in inhibiting the malignant behaviors of HCC cells maybe involve in activating the activity of caspase-3 and blocking the transduction of PI3K/AKT signaling pathway. Vinco is a natural drug that can be used alone or in combination with other drugs for treating HCC patients.

Abbreviations

HCC: Hepatocellular carcinoma; Vinco: Vincosamide; AFP: Alpha fetoprotein; TRAIL: Tumor necrosis-related apoptosis-induced ligand; PTEN: phosphate and tension homology deleted on chromosome ten.

Declarations

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Authors’ contributions

ML and MZ designed the experiments. MZ, HF, BL, YZ, YZ and KL performed the experiment. ML and MZ supervised the study, analysis the data. ML written the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this manuscript.

**Ethics approval and consent to participate**

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Hainan Medical College, Haikou, Hainan Province, PR. China.

**Consent for publication**

All authors have read and agreed to publish this manuscript.

**Competing interests**

All the authors confirm that no conflicts of interest are associated with the content of this article.

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Figures
Influence of Vinco on the growth and viability of the normal liver cell line L-02 and the HCC cell lines HLE, Bel 7402 and PLC/PRF/5. A, Molecular structure of Vinco (element components: C26H30N2O8; molecular weight: 498.532 Da). B, L-02 cells and the HCC cell lines HLE, Bel 7402 and PLC/PRF/5 were treated with different concentrations (5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml or 80 μg/ml) of Vinco for 48 h. The MTT assay was applied to detect the growth of the cells. *P<0.05 and **P<0.01 vs the control groups (0 μg/ml).
N=6. C and D, L-02, HLE, Bel 7402 and PLC/PRF/5 cells were treated with different concentrations (10 μg/ml or 80 μg/ml) of Vinco for 24h, 48 h, 72h, respectively, and CCK-8 assay was applied to detect the proliferation of the cells, *P<0.05 and **P<0.01. N=6. L-02, HLE, Bel 7402 and PLC/PRF/5 cells were treated with different concentrations (10 μg/ml or 80 μg/ml) of Vinco for 48 h, the trypan blue exclusion dye assay was used to analyze the viability of these cells, the bar graphs on the right show a quantitative of viability cells, P<0.05 indicating statistical significance (E); and the morphology of these cells was observed by microscopy (F); The nuclei of these cells were stained with DAPI and observed by fluorescence microscopy, the bar graphs on the right show a quantitative assessment of the frequency of nuclear morphological changes, P<0.05 indicating statistical significance (G). The red arrows indicate cellular nucleus condensation and pyknosis. The images are representative of at least three independent experiments.
Figure 2

Effects of Vinco on mitochondrial morphology in HLE, Bel 7402 and PLC/PRF/5 cells. HCC cells were treated with Vinco (80 μg/ml) for 12 h, and changes in the morphology of mitochondria in the cells were observed by electron microscopy. *Indicates death cells, and the white arrows indicate mitochondria. The images are representative of three independent experiments.
Figure 3

Influence of Vinco on scratch repair and migration, and the expression of metastasis-related proteins in HCC cells. A, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(10 ug/ml) for 24 h or 48 h. Scratch repair was observed by microscopy. The bar graph on the right shows the repair ratio of the cells, with P< 0.05 indicating statistical significance. B, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(10 ug/ml or 80 ug/ml) for 48 h. Migratory cells were stained with 0.1% crystal violet and observed
by microscopy. The lower bar graph show the number of migratory cells; with P<0.01 indicates statistical significance. C, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco (10 µg/ml or 80 µg/ml) for 48 h, and the expression of the metastasis-related proteins MMP9, CXCR4 and EpCAM were detected by Western blotting. The bar graphs on the right show the relative expressed quantity of these proteins, with P< 0.05 indicating statistical significance. The images are representative of three independent experiments.

Figure 4
Effects of Vinco on nuclear migration and activity of caspase-3, and the expression of apoptosis-related proteins in HCC cells. HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(10 μg/ml or 80 μg/ml) for 48 h, and the nuclear migration of caspase-3 molecules in these cells was observed by laser confocal microscopy (A). The expression of the apoptosis-related proteins Bax, Bcl-2, cleaved caspase-3 and PARP-1 was detected by Western blotting. The bar graphs on the right show the relative expression of these proteins. *P<0.05, **P<0.01 vs the untreated group (control) (B). The quantitative expression ratio of Bax/Bcl-2 (C), with P<0.05 indicates statistical significance. The images are representative of at least three independent experiments.

D, HLE, Bel 7402 and PLC/PRF/5 cells were treated with TRAIL(2 μmol/L) or Vinco(80 μg/ml) and a caspase-3 inhibitor (Z-DEVD-FMK) for 48 h. The activity of caspase-3 in these cells was measured using an enzymatic reaction Kit. **P<0.01, ***P<0.001 vs control group, ΔP<0.01 vs the control group, TRAIL(2 μmol/L)-treated group or Vinco(80 μg/ml)-treated group; ΔΔP<0.05 vs the TRAIL(2 μmol/L)- and Vinco(80 μg/ml)-treated group. N=6. The images are representative of at least three independent experiments.

Figure 5

The influence of Vinco on the expression of PTEN, and the PI3K/AKT signaling pathway is regulated by PTEN express in HCC cells. A, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(80 μg/ml) for 48 h, and the expression and localization of PTEN in these cells were observed by laser confocal microscopy. B, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco(10 μg/ml or 80 μg/ml) for 48 h, and the expression of PTEN, pAKT(Ser473), pmTOR(Thr2448) was detected by Western blotting. The bar graphs on the right show relative expressed quantity of these proteins, with P<0.05 indicating statistical significance; *P<0.05 and **P<0.01 vs control and Vinco(10 μg/ml)-treated groups. The images are representative of at least three independent experiments.
Figure 6

The influence of Vinco on the expression of the oncogene Src, the reprogramming gene Oct4, and the proteins related to the regulation by PI3K/AKT signaling pathway in HCC cells. HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco (80 μg/ml) for 48 h, and the expression and localization of Src(A) and Oct4(B) in these cells were observed by laser confocal microscopy. C, HLE, Bel 7402 and PLC/PRF/5 cells were treated with Vinco (80 μg/ml) or the PI3K inhibitor Ly294002 (2 μmol/L) or co-treated with...
Vinco (80 μg/ml) and Ly294002 (2 μmol/L) for 48 h, and the expression of pAKT (Ser473), Src, and Oct4 was detected by Western blotting. The bar graphs on the right showed relative expressed quantity of these proteins, with P<0.05 indicating statistical significance; **P<0.01 vs Ly294002- treated groups. The images are representative of at least three independent experiments.

Figure 7

The effect of Vinco on HCC cells growth, the expression of the growth-related factors PTEN, c-myc, Ras, and pAKT (Ser473), and the activity of caspase-3 in vivo. Bel 7402(A) and PLC/PRF/5(B) cells (1×10^6) in 0.1 ml of Hank balanced salt solution were subcutaneously injected into the right scapular region of male nude mice (n=7). The mice were intraperitoneally injected with Vinco (10 mg/kg/day) every day. Tumor-bearing mice were killed every 3 days after inoculation, the tumors were removed, and the measured length (L), weight (W) of the tumors were measured, and the volume (V) was calculated by formula by the following formula: V=π/6×L×W^2 (π=3.14). The lower bar graphs show tumorous volume. C and D, The
expression of c-myc, Ras, cleaved caspase-3 and PARP-1 in tumorous tissues (extracted from tumor-bearing mice on day 21) were detected by immunohistochemistry. E, The expression of cleaved caspase-3, PARP-1, PTEN and pAKT(Ser473) in tumorous tissues were detected by Western blotting. The bar graphs on the right show relative expressed quantity of these proteins. *P<0.05 and **P<0.01 vs the untreated group. The images are representative of at least three independent experiments.

Figure 8

Schematic of the mechanism underlying the role of Vinco in inhibiting the malignancy of HCC cells.