Hindawi Publishing Corporation
Journal of Nanomaterials
Volume 2013, Article ID 623497, 6 pages
http://dx.doi.org/10.1155/2013/623497

Research Article

Evaluation of the Antitumor Activity by Ni Nanoparticles with Verbascoside

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Received 1 September 2013; Revised 25 October 2013; Accepted 4 November 2013

Academic Editor: Mengnan Qu

Verbascoside (VB) has attracted a great deal of attention due to ITS pharmacological properties. In our study, we synthesized a multifunctional verbascoside coated Ni nanoparticles (VB-Ni). Transmission electron microscopy (TEM) and high performance liquid chromatography (HPLC) display the characteristics of VB-Ni nanoparticles. Compared with VB, VB-Ni has been proven to induce apoptosis and resist the growth of doxorubicin-resistant K562 cells *in vitro* and *in vivo*. Thus, VB-Ni nanoparticles can be thought of as an ideal mode of cancer treatment.

1. Introduction

Cancer is quickly becoming the leading cause of death worldwide [1]. Nickel nanoparticles (Ni NPs) have been applied in a wide range of fields due to their unique structure and properties [2–6]. Over the past decades, nanoparticles have been increasingly applied in clinical diagnoses and cancer therapy with promising and far-ranging prospects in the medical fields. Increasing interest in the application of nanotechnology for cancer therapy has been noted [7–10]. Previous phytochemical studies have demonstrated that flavonoids and phenylpropanoid glycosides are major bioactive constituents of the Tsoong herb (Chinese name: Banchunmaxianhao, BCM) [11]. Among these constituents, VB has attracted a great deal of attention due to its pharmacological properties [12–17]. Its properties include hepatoprotective, anti-inflammatory, antitumor, cytotoxic, and antioxidant activities [18–20].

In recent years, many studies on the therapeutic effect of drug-loaded nanoparticles have become a hot spot [21, 22]. Based on the above considerations, we have verified the biological effects of VB-Ni nanoparticles on treating cancer cells [23, 24]. These observations indicate their great potential in clinical and biomedical applications.

2. Materials and Methods

2.1. Materials. BCM were collected from Gangcha, QingHai, China, and identified by Professor Li-Juan Mei (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). Materials used for HPLC analysis were of analytical grade.

2.2. Cell Culture. K562 cells were purchased from Tianjin Institute of Hematology and cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% FBS (GIBCO) and penicillin (100 U/mL)/streptomycin (100 mg/mL) at 37°C in a 5% CO_2, water-saturated atmosphere. To test the function of VB-Ni, VB-Ni, or VB was added to K562 cells in the same concentration. Cells were

observed by microscope after 48 or 72 h treatment, using DNA Ladder to detect the apoptosis of cells.

2.3. Extract VB from BCM Plant. BCM (500 g) were powdered and extracted three times with 70% EtOH under reflux. After concentration under vacuum, the residues were suspended in distilled water and extracted with light petroleum, EtOAc, and n-butanol, respectively. The n-butanol solutions were evaporated to dryness under vacuum at 70°C to generate n-butanol extract, which was loaded on silica gel column and eluted with various proportions of a mixture of chloroform : methanol. The chloroform : methanol (3 : 1) fraction was concentrated to produce crude sample for subsequent high-speed counter-current chromatography (HSCCC) isolation and purification. With a two-phase solvent system composed of chloroform : n-butanol : methanol : water (4 : 3 : 4 : 5, v/v), the crude sample was separated to yield VB.

2.4. Preparation of Drug-Loaded Ni Nanoparticle. We mixed 2 mg VB and 30 mg Ni nanoparticles with ddH_2O in nitrogen environment. Then, we separated the final product from the mixture solution by magnet, washed them for three times, and added 300 mL distilled water to suspend. Finally, VB-Ni nanoparticles were measured by transmission electron microscope (TEM).

2.5. DNA Fragmentation Assay. K562 cells were incubated with VB, Ni, or VB-Ni for 24 h, 48 h, and 72 h, respectively. The untreated cells served as controls. DNA was extracted from K562 cells using Apoptotic DNA ladder isolation kit (Yuan Ping Hao Biotechnology Co., Ltd, Beijing, China), and then loaded onto 1% agarose gel. The DNA ladders stained with ethidium bromide were visualized under UV light.

2.6. Acridine Orange/Ethidium Bromide (AO/EB) Staining to Detect Apoptosis. K562 cells were incubated with VB-Ni for 48 h or 72 h. To stain apoptotic cells, the cells were trypsinized for 5 min before adding 1 μL of AO/EB dye mixture (100 μg/mL acridine orange and 100 μg/mL ethidium bromide) to each well. Then, cells were viewed under the fluorescent light microscope.

2.7. Experimental Animals. The female mice (6-week old) were purchased from the Animal Feeding Farm of National Institute for the Control of Pharmaceutical and Biological Products (China). All K562 tumor C57 mice were housed in the animal facility, and animal experiments, (1) control ($n = 3$), (2) VB ($n = 5$), (3) VB-Ni plus a magnet fixed under skin close to the tumor site ($n = 5$), that were conducted followed the guidelines by the Animal Research Ethics Board of Nanjing Medical University. Animals were kept in the facility with free access to food and water. Injection was intravenously administered by tail vein at days 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18. The tumor volume of mice was measured and calculated at the 20th day after treatment. The tumor volume calculation was performed using the formula ($\pi \times$ long axis $\times$ short axis $\times$ short axis)/6.

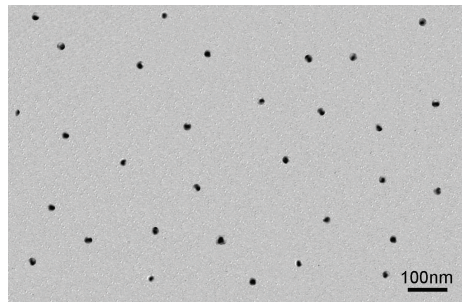

Figure 1: TEM images of VB-Ni nanoparticle. TEM image of an individual nanocrystal of VB-Ni.

3. Results

3.1. Synthesis and Characterization of VB-Ni Nanoparticles. In our previous study, the structure of VB has been identified and synthesized through HPLC analysis. And the NMR data of VB was in agreement with published data [25]. Herein, we further measured the size of VB-Ni nanoparticle, which was synthesized successfully by mixed 2 mg VB with 30 mg Ni in solution. As shown in Figure 1, the average diameter of VB-Ni nanoparticle was about 10 nm in TEM image. The size of VB-Ni nanoparticles was about 15 nm in cell culture medium through particle sizer analysis, which was relatively uniform and stable (unpublished data).

3.2. The Effects of VB-Ni in Recipient K562 Cells. Many researchers including us have reported that VB components can increase the apoptosis and inhibit the growth of cancer cells *in vitro* and *in vivo* [26]. As a new nanoparticle, we also determined the effect of VB-Ni in doxorubicin-resistant K562 cells. Firstly, K562 cells were incubated for 24 hours with the same concentration of VB and VB-Ni. We found that the apoptosis level was higher in VB-Ni group than VB group (Figures 2(A), 2(b), and (c)). However, to study the relationship between time and the enhanced effect of apoptosis in K562 cells, we treated the cells with VB-Ni at different time points. We further detected the apoptosis level of K562 cells after treatment of VB-Ni. The result demonstrated that the apoptosis rate of K562 cells was further enhanced after 72 h treatment than 24 h (Figures 2(A) and (d)). Consistent with our hypothesis, VB-Ni was effective to increase the apoptosis of cancer cells. Besides, we observed a similar inhibitory effect of VB-Ni on viability or survival in K562 cells through MTT assay (Figure 2(B)).

3.3. Fluorescence Microscopic Assay of Apoptosis in K562 Cells. In order to further determine the apoptosis effect of VB-Ni, next, we treated K562 cells with VB-Ni. Using AO/EB staining for apoptotic cells, apoptotic nuclei were identified by their characteristic features such as chromosomal condensation, distinctively marginated, and fragmented under fluorescence

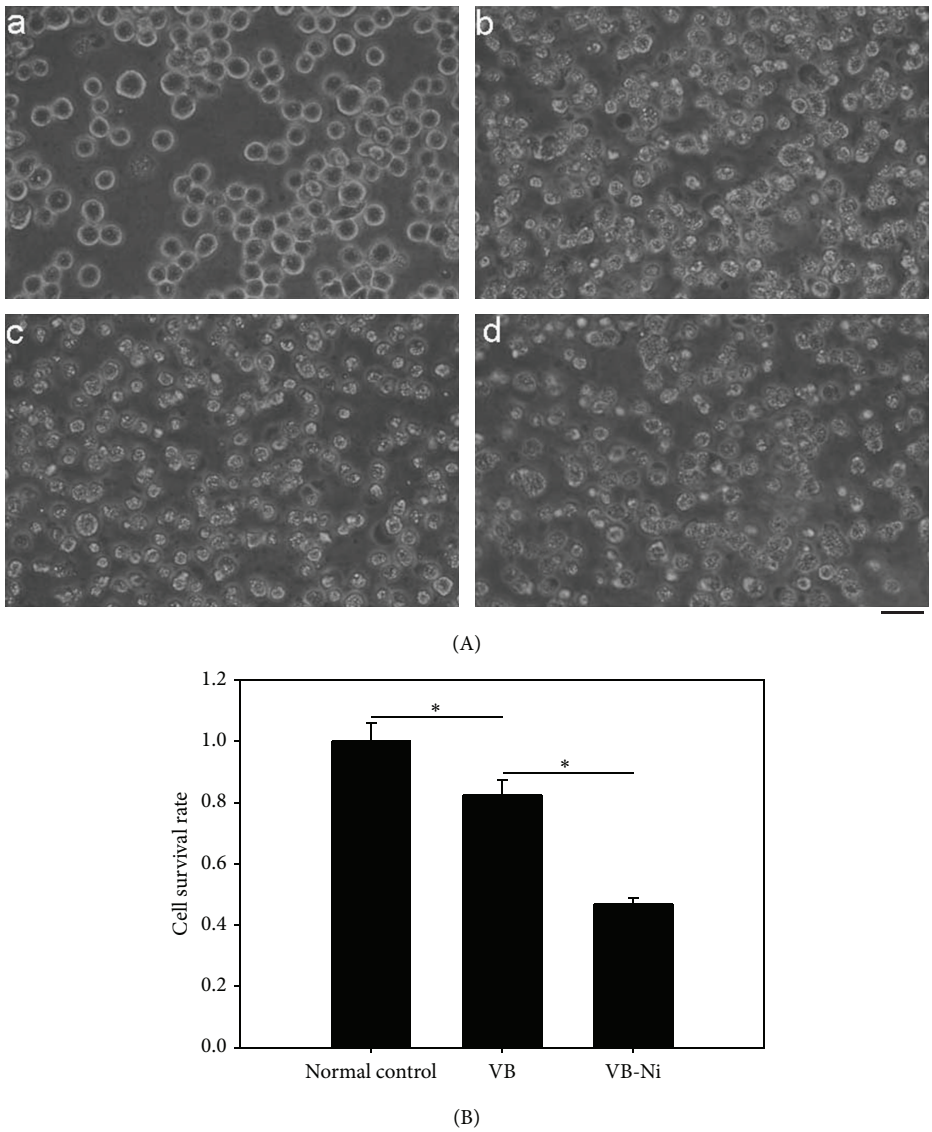

Figure 2: The effects of VB-Ni in recipient K562 cells. (A) K562 cells were treated with or without VB-Ni, VB for 48 h or 72 h, and then observed under microscopy. (a) Microscopy image of normal K562 cells. (b) K562 cells were treated with VB for 48 h. (c) K562 cells were treated with VB-Ni for 48 h. (d) K562 cells were treated with VB-Ni for 72 h. (scale bar = 100 nm). (B) MTT measurement of cellular survival in K562 cells treated with VB or VB-Ni.

microscope. We found that the apoptotic nuclei in K562 cells treatment with VB-Ni (Figures 3(c) and 3(d)) were emerged compared with normal control (Figures 3(a) and 3(b)).

3.4. DNA Fragmentation Experiments. To figure out whether the cell growth inhibition was caused by the apoptotic response, the DNA fragmentations were examined by agarose gel electrophoresis. When K562 cells were treated with VB-Ni or VB (Figure 4), the intensity of fragmented chromosomal DNA bands was much higher than that observed from cells untreated (Figure 4, lanes 1 and 2, resp.) in a fixed time interval (i.e., 24 h (a), 48 h (b), and 72 h (c)). The formation of DNA ladders was clearly present after treatment with VB-Ni (Figure 4, lane 1) but was only weakly discernible when the cells were treated with VB (Figure 4, lane 2). Our observations support the hypothesis that the remarkable enhancement of apoptosis was induced by the synergistic effect of VB-Ni nanoparticles on K562 cells.

3.5. VB-Ni Inhibits the Tumor Growth In Vivo. We next tested whether delivery of VB-Ni can efficiently suppress the implanted tumor growth in mice. In this experiment, C57 mice were subcutaneously implanted with K562 tumor cells. Mice were intravenously injected with different reagents every other day. After 20 days, the treatment with VB-Ni effectively reduced the volume (Figures 5(a) and 5(b)) or weight (Figure 5(c)) of the implanted tumors compared with VB-treated control. These data strongly suggest that the VB-Ni can transfer Ni into the mouse implanted tumor cells, in

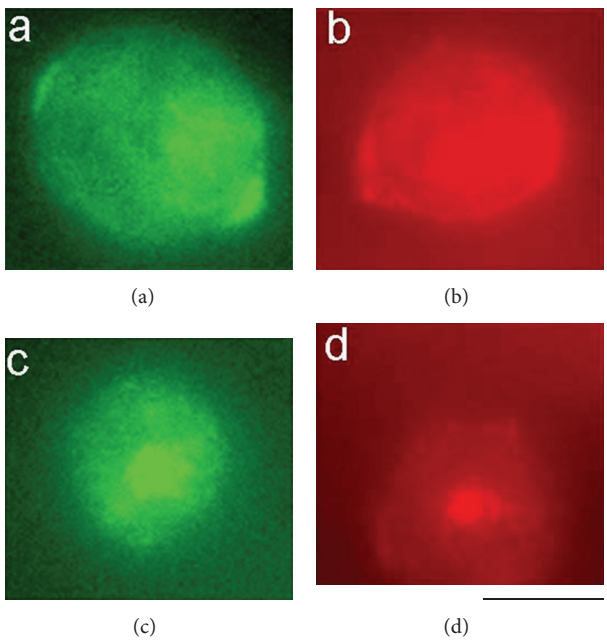

Figure 3: Fluorescence detection of the apoptosis of K562 cell. Detect the normal and apoptotic K562 cells by Acridine orange/ethidium bromide (AO/EB) staining. (a) Early apoptotic nuclei of normal control K562 cells were observed. (b) Later apoptotic nuclei of normal control K562 cells were observed. (c) Early apoptotic nuclei of treatment K562 cells with VB-Ni were observed. (d) Later apoptotic nuclei of treatment K562 cells with VB-Ni were observed (400x). (bar = 10 μm).

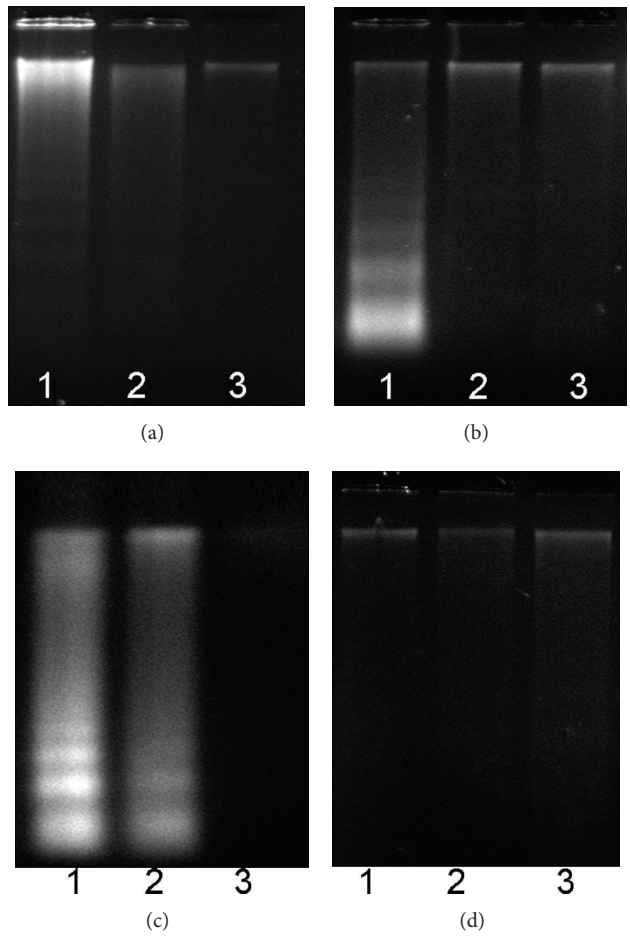

Figure 4: DNA fragmentation in K562 cells after different treatments. Genomic DNA was extracted from K562 cells treated with various regents using Apoptotic DNA ladder isolation kit and then loaded onto 1% agarose gel. Then, DNA ladders were visualized under UV light with ethidium bromide staining. (a) K562 cells were treated with VB-Ni (lane 1), VB (lane 2), or control (lane 3) treatment for 24 h. (b) K562 cells were treated with VB-Ni (lane 1), VB (lane 2), or control (lane 3) treatment for 48 h. (c) K562 cells were treated with VB-Ni (lane 1), VB (lane 2), or control (lane 3) treatment for 72 h. (d) Normal K562 cells.

which Ni with VB suppresses the tumor cell growth (plus a magnet fixed under skin close to the tumor site).

4. Discussion

In this study, we demonstrated that a combination of verbascoside (VB) and Ni where the VB is bound to Ni surface by electrostatic interaction will suppress the growth of tumor cells. Compared with VB-Ni, the same or even higher concentration of VB did not cause a significant reduction in cell viability in K562 cells. However, when K562 cells were treated with VB-Ni, we observed a remarkable enhancement of cell growth inhibition (Figure 2). The results strongly suggest that the VB-Ni nanoparticles can induce cell growth inhibition of K562 cell *in vitro*.

Two major types of cell death are recognized: apoptosis and necrosis [27]. Apoptosis is a regulated process that can be triggered by different stimuli and is mediated by a cascade of enzymes. Necrosis is a catastrophic form of cell death which does not involve the regulated action of enzymes. Studies have demonstrated that the presence of smaller DNA fragments is believed to reflect the release of nucleosomes from apoptotic cells and higher molecular weight DNA molecules are believed to reflect release from necrotic cells. Apoptosis results in fragmentation of cells into apoptotic bodies which are engulfed by neighboring cells and macrophages. However, uptake of necrotic cells has been reported to be less efficient than phagocytosis of apoptotic cells. So active anticancer drugs which induce apoptosis in malignant cells should be a main way to clinical antitumor. Interestingly, we found that VB-Ni can induce K562 cell apoptosis with a rate significantly higher than that of VB, or Ni alone treatment *in vitro*. Moreover, we analyzed the cells apoptosis morphology from various assays, nuclei staining. When cells were treated with VB-Ni, they exhibited characteristic morphological features of apoptosis, such as chromosomal condensation and DNA fragment.

As the above results illustrated, we recognized the evidence of apoptosis of cancer cells *in vitro*. It is possible that VB-Ni could play a critical role in inducing apoptosis *in vivo*. The tumor growth in group 3 mice (treated with VB-Ni) (Figure 5(b)) was suppressed most efficiently.

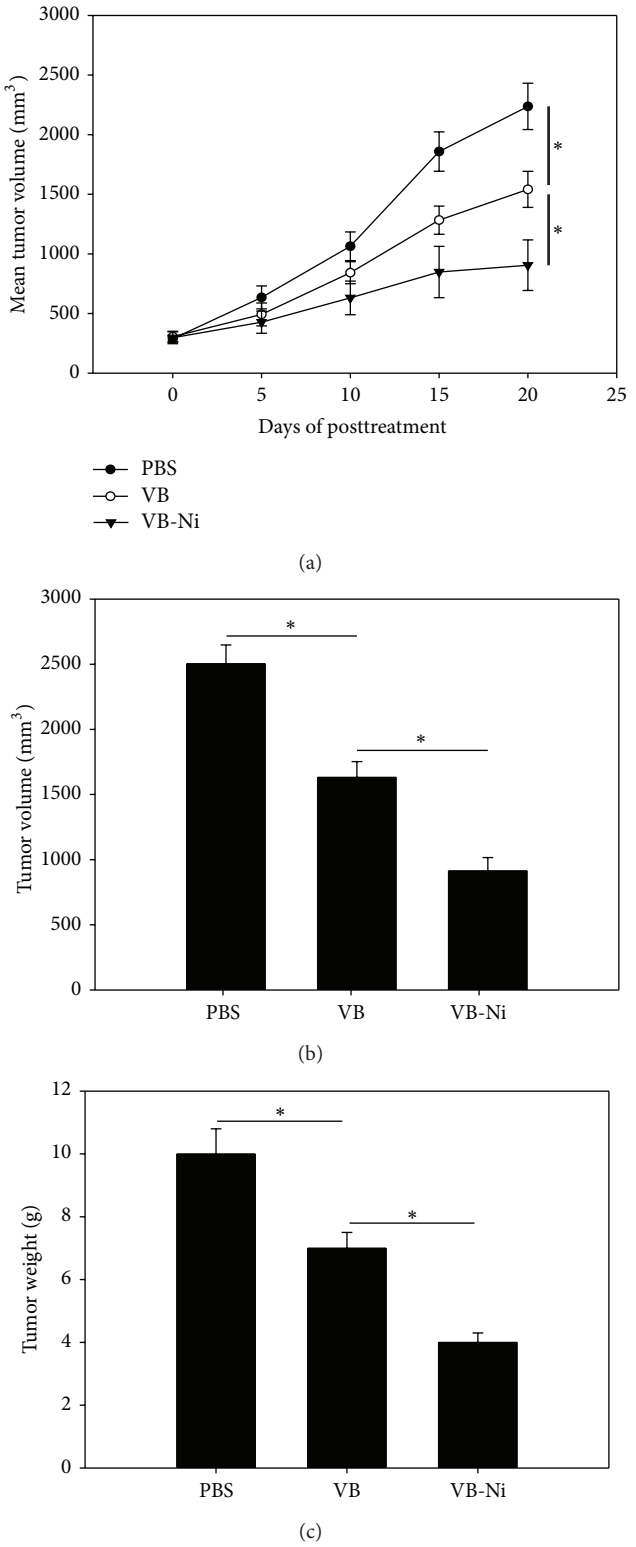

Figure 5: Inhibit the tumor growth in K652/ADM mice with VB-Ni treatments. (a) and (b) Tumor volumes in mice treated with various regents. Cancer cells were injected into the abdominal area of mice (plus a magnet fixed under skin close to the tumor site). When the tumors reached a minimal size of 300 mm^3, mice were divided to 3 groups with 3–5 mice in each group. The mouse groups received various reagents every other day by tail-vein injections. (c) The weight of tumor in (a).

5. Conclusion

In summary, in the current study, we have investigated the synergistic effect of Ni with the anticancer drug verbascoside (VB) on the induction of apoptosis of K562 cell. Our observations demonstrate that Ni readily facilitated the uptake of the VB into K562 cells by electrochemical assay. Apoptotic staining and DNA fragmentation further demonstrate that treatment of VB-Ni can clearly activate apoptosis in K562 cells. Moreover, our *in vivo* study indicates that the treatment of VB-Ni effectively inhibited the mice tumor growth. The increased cell apoptosis rate was closely correlated with the enhanced inhibition of tumor growth in the studied animals (plus a magnet fixed under skin close to the tumor site). Thus, VB-Ni may serve as a novel strategy to sensitively track the respective cancer cells for efficient cancer chemotherapy.

Authors' Contribution

Mingyue Chen, Yaqin Zhang, and Bin Huang contributed equally to this work.

Acknowledgments

This research was financially supported by the Priority Academic Program for the Development of Jiangsu Higher Education Institutions (JX10531801/005), Medical Science and Technology Foundation of Jiangsu, Department of Health (Z201008), NSFC (31201003), Funding from the State Key Laboratory of Bioelectronics of Southeast University, Nanjing Medical University Science and Technology Development Fund (KY10114311216122218 and KY10114311216122230), and Science and Technology Innovation Supporting Fund (JX10131801147, JX10131801149, and JX10131801154).

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