

RESEARCH COMMUNICATION

Efficient Killing Effect of Osteosarcoma Cells by Cinobufacini and Cisplatin in Combination

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Abstract

Purpose: To study the killing effects on osteosarcoma cells of cinobufacini and cisplatin in combination and the related mechanisms so as to explore the chemotherapeutic method with integrated traditional Chinese and Western medicines. **Methods:** Cinobufacini and cisplatin were applied to OS732 cells singly or jointly and survival rates were measured by MTT assay. Changes in cellular shape were observed with inverted phase contrast and fluorescence microscopy and apoptosis rates were analyzed with flow cytometry (FCM). Immunocytochemistry were used to examine the Fas expression of OS732 cells. **Results:** The combination of cinobufacini and cisplatin had the effect of up-regulating Fas expression and inducing apoptosis. The survival rate of combined application of 100 µg/ml cinobufacini and 1 µg/ml cisplatin on OS-732 cells was significantly lower than with either of the agents alone ($p < 0.01$). Changes in cellular shape and apoptotic rates also indicated the apoptosis-inducing effects of combined application were much enhanced. **Conclusion:** The combination of cinobufacini and cisplatin demonstrated strong killing effects on OS-732 cells which might be related to up-regulation of Fas expression.

Keywords: Osteosarcoma - cinobufacini - cisplatin - combined treatment - apoptosis

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Introduction

Osteosarcoma is the most common primary tumor of bone, and early pulmonary metastasis is liable to occur with bad prognosis (Bhurgri et al., 2009; Dotan et al., 2010; Broadhead et al., 2011; Limmahakhun et al., 2011). Although the chemotherapeutic agents like cisplatin has obvious killing effect on osteosarcoma cells, the resistance and toxic side effects after long-term application remain the main obstacle for clinical doctors. Cinobufacini, an aqueous extract from the skins of *Bufo bufo gargarizans* Cantor, is a well-known traditional Chinese medicine widely used in clinical cancer therapy in China and some Asian pacific countries (Cui et al., 2010; Wang et al., 2011; Chen et al., 2012). Currently, it has been proved clinically that cinobufacini may enhance the anti-tumor effect as well as reduce the doses of chemotherapeutic agents. However, there has been few reports about the application of cinobufacini in osteosarcoma chemotherapy (Li et al., 2006; Qi et al., 2008; Wang et al., 2009), therefore, we used cinobufacini and cisplatin respectively or jointly on OS732 cells so as to explore whether cinobufacini could induce cellular apoptosis and we also examined whether the effect was related with up-regulating the expression of Fas on osteosarcoma cells so as to analyze its anti-tumor mechanism and provide theoretic evidence for the integrated traditional Chinese and western medicine

treatment of osteosarcoma.

Materials and Methods

The osteosarcoma OS732 cell line was bought from Beijing Jishuitan hospital. RPMI-1640 powder were from the Gibco company. Trypsin, MTT were from Huamei biological company. Cinobufacini injection was provided by Anhui Jinchuan Biochemical Co., Ltd. and cisplatin by Qilu pharmaceutical factory. Multi-clone antibody of Fas was bought from Fuzhou Maixin biological company.

Cell culture and research methods

The OS732 cell line was placed in the 1640 culture solution with 10% fetal bovine serum and cultured in incubator at 37°C in a humidified 5% CO₂ atmosphere. The cells that entered the logarithmic growth period were selected for experiment.

We selected the concentrations of 1 µg/ml, 10 µg/ml, 100 µg/ml and 1000 µg/ml for the cinobufacini group, the concentrations of 1 µg/ml, 5 µg/ml, 10 µg/ml and 100 µg/ml for the cisplatin group, and 100 µg/ml cinobufacini with 1 µg/ml cisplatin for the combined group, meanwhile setting an additional PBS blank control group. The experiment times were as follows: 24 h, 48 h, 72 h, after the treatment with different drugs and all trials were repeated three times.

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Measurement of the survival rates of tumor cells with MTT method

5×10^5 /ml of cells were seeded in the 96-well plate with 200 μ l each well, and added to the culture medium containing agents of different concentrations or control PBS with 100 μ l each well, each concentration for parallel 4 wells after adherence. After culture for 24 h, newly made-up 20 μ l MTT was added, and continued to incubate at 37 °C for 4 h, then the supernatant was discarded and dissolved in 150 μ l DMSO. The absorbance was measured at 540 nm wavelength after mixed. Survival rate of tumor cells (%) = experimental group A value/control group A value \times 100%.

Observation of the morphology of apoptotic cells

The morphology, number and adherence of tumor cells were directly observed with inverted phase contrast microscope. A cover slide was placed in the 6-well plate, and after the apoptosis of cells, they were fixed for 10min, stained with 0.5 ml Hoechst33258 staining solution for 5 min, and then camera-imaged with fluorescence microscope on the object slide covered by cover slide and dropped with anti-fading solution.

Measurement of the proportion of apoptotic cells with flow cytometer(FCM)

The cells were added with 70% cold ethanol to fix for 48 h. After centrifugation with supernatant discarded, they were added with 10 g/l RNase200 μ l, water bathed at 37 °C for 30 min, washed out RNase, and stained in darkness with 100 μ lPI staining solution at 4 °C for 1 h. The fluorescence strength was measured with FACScan flow cytometer. The wavelength of activated light was 488 nm, and the apoptotic rates were measured with CellQuest analysis software.

Immunocytochemistry to detect Fas expression of OS732 cells

2×10^5 /ml of digestive cells were placed in a 6-well culture plate with pre-treated cover slide in each well. cultured for 24 h and then supernatant were discarded, added with medicine, continue to culture for 24 hours ,meanwhile set blank control group,The cover slide were removed, fixed with acetone at 4 °C for 10 min and stained with SP method according to manual. The brown yellow cytoplasm indicated positive, and the expression intensity of Fas was inversely determined by the average gray value obtained with image analysis system, which means the more is the average gray value, the less is the Fas level.

Statistic method

Experimental data were given as means \pm standard deviations (SDs), compared between different groups by t-test with WindowsSPSS13.0 software.

Results

Changes of survival rates of tumor cells

When the concentration of cinobufacini was 1 μ g/ml, 10 μ g/ml, 100 μ g/ml and 1000 μ g/ml, the cell growth

was inhibited in a dose-dependent manner. There were significant differences between different groups ($P < 0.05$) (Figure 1A). Similarly, when the concentration of cisplatin was 1 μ g/ml, 5 μ g/ml, 10 μ g/ml and 100 μ g/ml, the cell growth was also suppressed between different groups ($P < 0.05$) (Figure 1B). With the combination of 100 μ g/ml cinobufacini and 5 μ g/ml cisplatin, the survival rate was significantly lower ($P < 0.01$) (Figure 1C), compared with the respective use of 100 μ g/ml cinobufacini or 1 μ g/ml cisplatin, showing that the combined use of cinobufacini and cisplatin may have stronger inhibition effect than the single agent. More importantly, we found that cell growth was inhibited in a time-dependent manner since the survival rate of OS732 cells were the lowest at 72 h and the highest at 24 h.

Morphological changes of apoptosis of OS-732 cells

Under the inverted phase contrast microscope, The

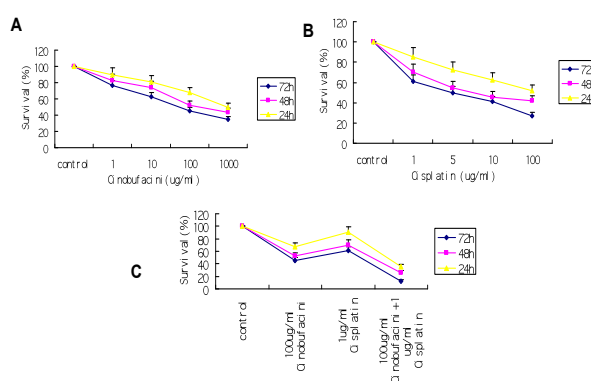


Figure 1. The Inhibition Effect on OS732 after 24h, 48h, 72h Measured by MTT. (A) The survival rate of OS732 with different concentration of cinobufacini. (B) The survival rate of OS732 with different concentration of cisplatin. (C) The survival rate of OS732 with combination of 100 μ g/ml cinobufacini and 1 μ g/ml cisplatin

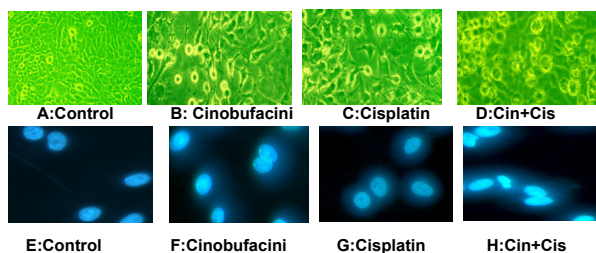


Figure 2. Morphological Changes and Fluorescent Staining of OS732 Cells after 72h with Different Drugs. (A) Morphological appearance of OS732 under inverted phase contrast microscope $\times 400$. (B) Morphological changes of OS732 treated with 100 μ g/ml cinobufacini under inverted phase contrast microscope $\times 400$. (C) Morphological changes of OS732 treated with 1 μ g/ml cisplatin under inverted phase contrast microscope $\times 400$. (D) Morphological changes of OS732 treated with combination of 100 μ g/ml cinobufacini and 1 μ g/ml cisplatin under inverted phase contrast microscope $\times 400$.

(E) Fluorescent staining of OS732 cells under the fluorescence microscope $\times 400$. (F) Fluorescent staining of OS732 cells treated with 100 μ g/ml cinobufacini under the fluorescence microscope $\times 400$. (G) Fluorescent staining of OS732 cells treated with 1 μ g/ml cisplatin under the fluorescence microscope $\times 400$. (H) Fluorescent staining of OS732 cells treated with 100 μ g/ml cinobufacini and 1 μ g/ml cisplatin under the fluorescence microscope $\times 400$

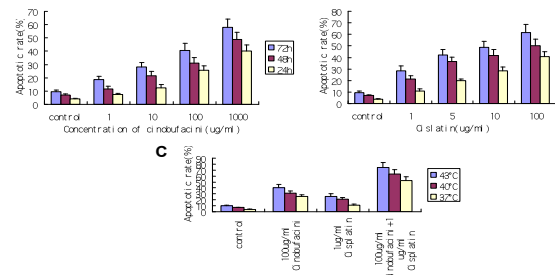


Figure 3. Apoptotic Effect of OS732 Cells after 24h, 48h, 72h Treated with Different Drugs. (A) Apoptotic rate of OS732 treated with different concentration of cinobufacini. (B) Apoptotic rate of OS732 treated with different concentration of cisplatin. (C) Apoptotic rate of OS732 treated with combination of 100 µg/ml cinobufacini and 1 µg/ml cisplatin

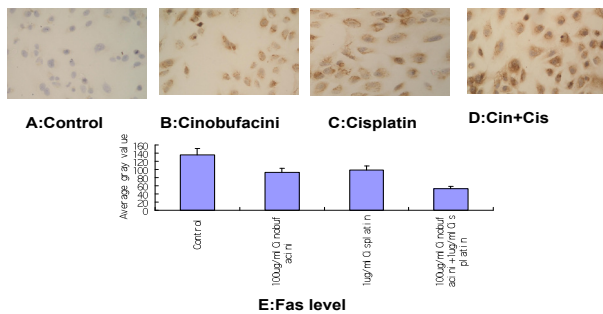


Figure 4. Fas Expression of OS732 Cells after 72h with Different Drugs. (A) Fas expression of OS732 by immunocytochemistry $\times 400$. (B) Fas expression of OS732 treated with 100 µg/ml cinobufacini by immunocytochemistry $\times 400$. (C) Fas expression of OS732 treated with 1 µg/ml cisplatin immunocytochemistry $\times 400$. (D) Fas expression of OS732 treated with combination of 100 µg/ml cinobufacini and 1 µg/ml cisplatin by immunocytochemistry $\times 400$. (E) Quantitative analysis of Fas level by comparing the average gray value of different groups with Meta Morph automatic image analyzer

normal OS-732 cells were attached to the dish, the cells were rhombus-like and angular (Figure 2A). with individual application of cinobufacini (100 µg/ml) or cisplatin (1µg/ml), only part of the cells became small and round (Figure 2B, 2C), However, with the combined application, chromatin and cytoplasm were condensed, and many cells became nonadherent and suspended in the culture medium (Figure 2D). Under fluorescence microscope, OS cells were lightly-stained (Figure 2E), whereas with individual application of cinobufacini (100 µg /ml) or cisplatin (1 µg/ml) , only only a few cells showed condensed and flared fluorescence (Figure 2F, 2G). With the combination of cinobufacini (100 µg/ml) and cisplatin (1 µg/ml), obvious condensed and flared fluorescence was observed, revealing the presence of many apoptotic cells (Figure 2H).

Comparison of apoptotic rates of OS-732 cells

After the treatment of the cells for 24h, 48h and 72h, with various concentration of cinobufacini and cisplatin, the apoptotic rate increased in a dosage-dependent manner. There were significant differences between the different groups ($P < 0.05$) (Figure 3A,3B). The apoptotic rate of the combination of cinobufacini (100 µg/ml) and cisplatin (1 µg/ml) is significantly higher than the individual application of cinobufacini (100 µg/ml) or cisplatin (1

µg/ml ($P < 0.01$) (Figure 3C).

Fas expression of OS732 by Immunocytochemistry

We observed only a small amount of brown particles in the cytoplasm of OS732 cells in the control group (Figure 4A). Deeper staining cytoplasm of OS732 cells with 100 µg/ml cinobufacini (Figure 4B) or 1 µg/ml cisplatin (Figure 4C); With the combination of 100 µg/ml cinobufacini and 1 µg /ml cisplatin, Deepest staining in cytoplasm, deformed, huge osteosarcoma cells is clearly visible, all Vision are covered with dye range (Figure 4D). we further measured the fas level quantitatively with Meta Morph automatic image analyzer by comparing average gray value which is Inversely proportional to the Fas expression (Figure 4E).

Discussion

Cinobufacini is a Chinese medicine prepared from the toad skin which has been extensively used in clinics to treat a number of diseases, such as malignant tumors (Chen et al., 2003; Dong et al., 2007; Qi et al., 2010a; Ma et al., 2012), chronic hepatitis B and systemic and local infection (Cui et al., 2010). However, little is known about its killing effect on osteosarcoma. Experimental pharmacodynamics and clinical study both indicate that cinobufacini has obvious killing effect on many kinds of tumor cells, and the mechanism may be related with the apoptosis-inducing effect on tumor cells (Qi et al., 2008; Qi et al., 2010b; Wang et al., 2010). Our research found that cinobufacini may inhibit the proliferation of OS-732 cells in a dose-dependent manner which was consistent with the above research results. Our results indicated, after 72 h of the treatment, when the concentration of cinobufotalin increased from 1 ug/ml to 1000 ug/ml, the survival rate only reduced from 76.28% to 34.55%, which revealed that Cinobufacini could inhibit the osteosarcoma cells, but osteosarcoma cell is not very sensitive to cinobufacini (Figure1A). We also found when the concentration of cisplatin increased from 1 ug/ml to 100 ug/ml, the survival rate reduced from 61.36% to 26.73 which indicated that osteosarcoma cells were more sensitive to cisplatin and the anti-tumor effect of cisplatin was stronger than cinobufacini (Figure1B). All these results were consistent with the main view in the clinical treatment that cisplatin is the first-line anticancer drugs for regular chemotherapy and the cinobufacini is the adjuvant second-line anticancer drugs which could be conducive to improve patient's immunity function so as to prevent the recurrence of tumor after the regular chemotherapy is over. According to our results, we deduce, cinobufacini is suitable for the treatment of osteosarcoma. To our surprise, after 72 h of combination of 100 ug/ml cinobufacini and 1 ug/ml of cisplatin on OS732 cell line, the survival rate dropped sharply to 12.65%. Obviously, the synergistic-killing effect is much stronger than the individual use of 100 ug/ml cinobufacini or 1 ug/ml cisplatin ($P < 0.01$, Figure1C). This suggested us that cinobufacini could enhance killing effect of osteosarcoma cells when combined with cisplatin, therefore, a greater anti-tumor effect would be triggered if cinabufotalin were

used in the chemotherapy. Since there were a great number reports about side effects on digestive, nervous, blood and other body systems due to application of cisplatin during the regular chemotherapy of osteosarcoma patients (Konstantakou et al., 2009; LaPensee et al., 2010; Maroto et al., 2011; Rotte et al., 2010; Sprowl et al., 2012), we suppose combination of cinobufacini and cisplatin will avoid all these side effects of chemotherapy, and by this way, cinobufacini will promote from the second-line to the first-line anti-tumor drugs and will play a more important role in osteosarcoma therapy. In addition, our research showed the killing effect by the application of cinobufotalin and cisplatin were fulfilled by inducing apoptosis of osteosarcoma cells which were revealed from the cell morphology (Figure2A-2D), cell apoptosis (Figure2E -2H) and FCM analysis (Figure3A-3B).

Fas is a kind of trans-membrane protein, belonging to the super-family of TNF receptors (Moulian and Berrih-Aknin, 1998; O'Callaghan et al., 2008; Inoue et al., 2009; Matsuda et al., 2009; Akimzhanov et al., 2010; Liedtke and Trautwein, 2012), and can be expressed extensively on many normal and tumor tissues. When it is ligated with Fas antibody or ligand, the apoptotic signal may be transferred into cells, and the apoptosis of cells triggered by Fas pathway may be induced. Many anti-tumor agents induce the apoptosis of tumor cells by up-regulating the expression of Fas (Okamoto et al., 2006; Wei et al., 2006; Kim et al., 2007; Dai et al., 2009). Our research indicated 72 hours after the combination of 100 ug/ml cinobufotalin and 1ug/ml cisplatin, the apoptosis rate is as high as 74.63 percent, far higher than individual application of 100 ug/ml cinobufotalin or 1 ug/ml cisplatin (Figure3C), meanwhile, our results revealed that Fas expression of OS732 cell line improved significantly in combination group compared with respective administration (Figure4A-4E). All these results indicated that such a synergistic effect is probably achieved through triggering Fas Pathway. Currently, Many chemotherapy drugs, including cisplatin, were reported to induce apoptosis by up-regulating Fas expression (Shiu et al., 2007). As far as our research is concerned, the combined application of cinobufacini and cisplatin significantly increased the Fas expression of OS732 cells, therefore, we deduce that Fas-FADD pathway plays a critical role in achieving this synergistic effect. After all, the exact mechanism is yet up to deep research and verification.

In conclusion, cinobufacini seems to have prospective view concerning about the treatment of osteosarcoma. Although the individual application of cinobufacini on chemotherapy may still have some limitation, cinobufacini is suitable for the osteosarcoma therapy as a kind of sensitivity-enhancing agents of chemotherapy by combination with cisplatin. Our research indicated cinobufacini could not only increase the anti-tumor effect of cisplatin by overcoming the resistance to cisplatin but also suppress the side effects of chemotherapy by decreasing the dose of cisplatin. Moreover, our results also showed, to some extent, individual application of cinobufacini could also inhibit the osteosarcoma cells which suggested us it could be used respectively in preoperative and postoperative or unconventional chemotherapeutic course,

in order to enhance the immunity of patients as an adjuvant treatment. Therefore we believe cinobufacini will be a prospective anti-tumor method worthwhile to spread in the field of clinical treatment on osteosarcoma .

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References

- Akimzhanov AM, Wang X, Sun J and Boehning D (2010). T-cell receptor complex is essential for Fas signal transduction. *Proc Natl Acad Sci USA*, **107**, 15105-10.
- Bhurgri Y, Usman A, Bhurgri H, et al (2009). Primary malignancies of bone and cartilage in Karachi. *Asian Pac J Cancer Prev*, **10**, 891-4.
- Broadhead ML, Dass CR, Choong PF (2011). Systemically administered PEDF against primary and secondary tumours in a clinically relevant osteosarcoma model. *Br J Cancer*, **105**, 1503-11.
- Chen Z, Chen HY, Lang QB et al (2012). Preventive effects of jiedu granules combined with cinobufacini injection versus transcatheter arterial chemoembolization in post-surgical patients with hepatocellular carcinoma: a case-control trial. *Chin J Integr Med*, **18**, 339-44.
- Chen Z, Zhai XF, Su YH, et al (2003). Clinical observation of cinobufacini injection used to treat moderate and advanced primary liver cancer. *Zhong Xi Yi Jie He Xue Bao*, **1**, 184-6 (in Chinese).
- Cui X, Inagaki Y, Xu H, et al (2010). Anti-hepatitis B virus activities of cinobufacini and its active components bufalin and cinobufagin in HepG2.2.15 cells. *Biol Pharm Bull*, **33**, 1728-32.
- Dai ZJ, Gao J, Ji ZZ, et al (2009). Matrine induces apoptosis in gastric carcinoma cells via alteration of Fas/FasL and activation of caspase-3. *J Ethnopharmacol*, **123**, 91-6.
- Dong HJ, Zhang LJ, Zhai XF, et al (2007). Effects of different nursing methods in treatment of phlebitis caused by cinobufacini injection. *Zhong Xi Yi Jie He Xue Bao*, **5**, 585-7 (in Chinese).
- Dotan A, Dadia S, Bickels J, et al (2010). Expandable endoprosthesis for limb-sparing surgery in children: long-term results. *J Child Orthop*, **4**, 391-400.
- Inoue S, Harper N, Walewska R, Dyer MJ, Cohen GM (2009). Enhanced Fas-associated death domain recruitment by histone deacetylase inhibitors is critical for the sensitization of chronic lymphocytic leukemia cells to TRAIL-induced apoptosis. *Mol Cancer Ther*, **8**, 3088-97.
- Kim JS, Lee YC, Nam HT, et al (2007). Apiculan A induces cell death through Fas ligand up-regulation and microtubule disruption by tubulin down-regulation in HM7 human colon cancer cells. *Clin Cancer Res*, **13**, 6509-17.
- Konstantakou EG, Voutsinas GE, Karkoulis PK, et al (2009). Human bladder cancer cells undergo cisplatin-induced apoptosis that is associated with p53-dependent and p53-independent responses. *Int J Oncol*, **35**, 401-16.
- LaPensee EW, LaPensee CR, Fox S, et al (2010). Bisphenol A and estradiol are equipotent in antagonizing cisplatin-induced cytotoxicity in breast cancer cells. *Cancer Lett*, **290**, 167-73.
- Liedtke C, Trautwein C (2012). The role of TNF and Fas dependent signaling in animal models of inflammatory liver injury and liver cancer. *Eur J Cell Biol*, **91**, 582-9.

- Limmahakhun S, Pothacharoen P, Theera-Umpon N, et al (2011). Relationships between serum biomarker levels and clinical presentation of human osteosarcomas. *Asian Pac J Cancer Prev*, **12**, 1717-22.
- Li XY, Lu Y, Shan QX and Xia Q (2006). Effect of cinobufacini on vascular contractile of rat thoracic aorta. *Zhejiang Da Xue Xue Bao Yi Xue Ban*, **35**, 178-81 (in Chinese).
- Ma L, Song B, Jin H, et al (2012). Cinobufacini induced MDA-MB-231 cell apoptosis-associated cell cycle arrest and cytoskeleton function. *Bioorg Med Chem Lett*, **22**, 1459-63.
- Maroto P, Huddart R, Garcia del Muro X, et al (2011). Brief report: phase II multicenter study of temozolomide in patients with cisplatin-resistant germ cell tumors. *Oncology*, **80**, 219-22.
- Matsuda N, Yamamoto S, Takano K, et al (2009). Silencing of fas-associated death domain protects mice from septic lung inflammation and apoptosis. *Am J Respir Crit Care Med*, **179**, 806-15.
- Moulian N and Berrih-Aknin S (1998). Fas/APO-1/CD95 in health and autoimmune disease: thymic and peripheral aspects. *Semin Immunol*, **10**, 449-56.
- O'Callaghan G, Kelly J, Shanahan F, Houston A (2008). Prostaglandin E2 stimulates Fas ligand expression via the EP1 receptor in colon cancer cells. *Br J Cancer*, **99**, 502-12.
- Qi FH, Li AY, Lv H, et al (2008). Apoptosis-inducing effect of cinobufacini, Bufo bufo gargarizans Cantor skin extract, on human hepatoma cell line BEL-7402. *Drug Discov Ther*, **2**, 339-43.
- Qi FH, Li AY, Zhao L, et al (2010b). Apoptosis-inducing effect of cinobufacini on human hepatoma cell line HepG2 and its mechanism of action. *Yao Xue Xue Bao*, **45**, 318-23 (in Chinese).
- Qi F, Li A, Zhao L, et al (2010a). Cinobufacini, an aqueous extract from Bufo bufo gargarizans Cantor, induces apoptosis through a mitochondria-mediated pathway in human hepatocellular carcinoma cells. *J Ethnopharmacol*, **128**, 654-61.
- Okamoto K, Fujisawa J, Reth M, Yonehara S (2006). Human T-cell leukemia virus type-I oncoprotein Tax inhibits Fas-mediated apoptosis by inducing cellular FLIP through activation of NF-kappaB. *Genes Cells*, **11**, 177-91.
- Rotte A, Garmann D, Buss I, Jaehde U (2010). Effect of extracellular ATP on cisplatin-induced cytotoxicity in human ovarian carcinoma cells. *Chemotherapy*, **56**, 1-8.
- Shiu LY, Chang LC, Liang CH, et al (2007). Solamargine induces apoptosis and sensitizes breast cancer cells to cisplatin. *Food Chem Toxicol*, **45**, 2155-64.
- Sprowl JA, Gregorc V, Lazzari C, et al (2012). Associations Between ABCC2 Polymorphisms and Cisplatin Disposition and Efficacy. *Clin Pharmacol Ther*.
- Wang DL, Qi FH, Xu HL, et al (2010). Apoptosis-inducing activity of compounds screened and characterized from cinobufacini by bioassay-guided isolation. *Mol Med Report*, **3**, 717-22.
- Wang SS, Zhai XF, Li B (2009). Effects of cinobufacini injection on contents of serum thyroid-stimulating hormone and adrenaline in rats. *Zhong Xi Yi Jie He Xue Bao*, **7**, 228-31 (in Chinese).
- Wang SS, Zhai XF, Li B (2011). Effect of cinobufacini injection on the tumor growth of tumor bearing rats of different constitutions. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, **31**, 1101-3 (in Chinese).
- Wei X, Li CG, Nong S, Zhu XY, Huang XM (2006). The influence of *Solanum lyratum* Thunb extract on apoptosis and the expression of fas/fasL genes in HeLa cells. *Zhong Yao Cai*, **29**, 1203-6 (in Chinese).