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# Blockade of VEGF receptor signal pathway and antitumor activity of ON-III, a component from Chinese herbal medicine*

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**Running title:**

ON-III inhibited VEGF receptor tyrosine kinase

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**Abbreviations:**

VEGFR, Vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase; HDMEC, human dermal microvessel endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; HEPES, N-(2-Hydroxy ethyl)-Piperazine ethane Sulfonic acid; PMSF, phenylmethylsulfonyl fluoride

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## Abstract

Anti-angiogenesis is a promising strategy of cancer treatment. Vascular endothelial growth factor receptor (Flk/KDR) is a tyrosine kinase receptor and has been strongly implicated in tumor angiogenesis. In this study, we reported that ON-III (2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone) extracted from Traditional Chinese Medicine *Cleistocaly xoperculatus* dry flower reversibly inhibited KDR tyrosine kinase phosphorylation, but EGFR tyrosine kinase phosphorylation was unaffected under the same concentrations of ON-III. ON-III also inhibited MAPK and AKT activation of KDR signal transduction downstream molecules without reduced total MAPK and AKT. The results in vitro showed that ON-III inhibited growth of human vascular endothelia HDMEC cells in the presence of VEGF preferentially, compared with in the presence of EGF. Systemic administration of ON-III at nontoxic doses in nude mice resulted in inhibition of subcutaneous tumor growth of human hepatocarcinoma Bel7402 and lung cancer GLC-82 xenografts. The tumor vessel density decreased by immunohistochemical staining for CD31 after ON-III treatment. These results indicated that ON-III inhibited KDR tyrosine kinase, shut down KDR-mediated signal transduction and inhibited tumor growth of human xenografts in vivo.

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## Introduction

Angiogenesis, the formation of new blood vessels by sprouting from pre-existing endothelium, is a significant component of a wide variety of biological processes, including embryonic vascular development and differentiation, wound healing, organ regeneration, and pathological processes including tumorigenesis(Eberhard et al., 2000; Folkman, 1990). Many growth factors involve this process. Of the numerous growth factors and cytokines that have been shown to have angiogenic effects, VEGF appears to be a pivotal factor in pathological situations that involve neovascularization(Foekens et al., 2001; Smith et al., 2000). VEGF is produced by normal and transformed cells and plays a key role in the physiology of normal vasculature and in tumor-induced angiogenesis, which makes it important to understand the mechanisms through which this mitogen promotes cell proliferation(Nakopoulou et al., 2002).

VEGF firstly binds to either of two tyrosine kinase receptors, Flk1/KDR or Flt1. Signaling by such receptors facilitates activation of the intrinsic tyrosine kinase followed by autophosphorylation of tyrosine residues in the cytoplasmic signaling molecules that connect the activated receptor to transduction cascades and promote cellular responses (Colavitti et al., 2002; Dias et al., 2002; Geng et al., 2001). KDR receptor possesses intrinsic tyrosine kinase activity that is stimulated after ligand binding and receptor dimerization and is mandatory for transmission of a cytoplasmic signaling response(Binetruy-Tournaire et al., 2000; Zhu et al., 2001). The activation of KDR has also been shown to correlate with tumor growth, lymph node metastasis, and resistance to chemotherapy in many kinds of tumors(Baek et al., 2000; Thakker et al., 1999). Binding of VEGF to KDR receptor on the surface of endothelial cells facilitates its autophosphorylation of the PTK domain(Ichikura et al., 2001; Takahashi et al., 1999; Zhang et al., 2002). Activation of KDR by VEGF results in activation of PI3K/AKT, MAPK and PKC with the ultimate cellular response being DNA synthesis and cell proliferation. Vascular endothelial growth factor receptors (VEGFRs) are upregulated on

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endothelium at sites of active angiogenesis, providing an opportunity for selective therapeutic intervention (Hanahan, 1997; Zhang et al., 2002).

To exploit targeting at KDR tyrosine kinase for cancer treatment, we have been actively pursuing small molecule therapeutic strategies targeted at KDR receptor-mediated signal transduction pathway (Spiekermann et al., 2002; Wedge et al., 2002). In this report, we describe that ON-III, which is one of chalcone derivatives from Traditional Chinese Medicine *Cleistocaly xoperculatus* dry flower, could inhibit tyrosine phosphorylation of KDR and growth of human cancer xenografts. Although some synthetic molecules have been reported to inhibit the VEGFR kinase, this is a low molecular weight inhibitor of the VEGF/VEGF receptor system reported to be active at inhibiting VEGF-mediated processes from Traditional Chinese Medicine.

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## Materials and methods

**Cell Culture and Reagents.** The human dermal microvessel endothelial cells (HDMEC) were prepared as previous description(Qian et al., 1999). HDMEC cells, human lung adenocarcinoma GLC-82 and heptocarcinoma cell line Bel7402 cells were cultivated in RMPI1640 medium (Life Technologies, Inc.)(Zhu et al., 2000). Cells were grown in an incubator at 37℃ under 5% CO2 in air. KDR antibody, anti-EGFR antibody, anti-tyrosine antibody (PY99) was obtained from Santa Cruz. Anti-Akt, phospho-Akt, MAPK and phospho-MAPK antibody were purchased from Cell Signaling Co.

**Extraction and Isolation.** ON-III, 2', 4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone, was extracted and isolated from Traditional Chinese Medicine *Cleistocaly xoperculatus* dry flower. Its structure is shown in Fig. 1. The following is the steps of extraction and isolation of ON-III. The fresh buds (5.0 kg) of *Cleistocalyx operculatus*, collected at South China Botanical Garden, Guangzhou, China, were extracted with 95% EtOH three times at room temperature. The EtOH extract, after concentration *in vacuo*, was suspended in $H_2O$ and the aqueous suspension was sequentially extracted three times each with ether, EtOAc, and *n*-BuOH. The combined EtOAc solution, upon evaporation, yielded a brown syrup (13.0 g). This syrup was subjected to silica gel column chromatography, eluted with $CHCl_3$–MeOH mixtures of increasing polarities (99.5:0.5 to 97:3). The fractions obtained on elution with $CHCl_3$–MeOH (99.5:0.5) were combined and concentrated to afford a brown residue. This residue, upon recrystallization in benzene, yielded compound (690 mg): orange needles, mp 124-126 °C. The compound was identified by co-TLC with an authentic sample and by direct comparison of its spectral data with those reported(Zhang F, 1990).

**Immunoblot Analysis.** Cells after different treatment were placed on ice, and washed with ice-cold PBS twice. Cell lysates were harvested in lysis buffer with protein inhibitors.

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(lysis buffer: 50mM HEPES, 150mM NaCl, 10% Glycerol, 1% Triton, 1.5mM $MgCl_2$, 1mM EGTA. Protein inhibitos: 10ug/ml tryin, 10ug/ml aprotinin, 10ug/ml leuprptin, 1mM phenylmethylsulfonyl fluoride (PMSF), 200uM $Na_3VO_4$, 100mM NaF). After 10 minutes of 14,000g centrifugation, the supernatant was transferred to a clean microfuge tubes, protein was determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were then boiled in 5X sample buffer for 5 minutes at 90°C, and resolved on 10% Tris-glycin gels (Novex, San Diego, CA). After electrophoresis, protein was electotransferred to nitrocelluose membrane (Amersham, Arlington Heights, IL). Membrane was blocked with 5% milk in TBST( 10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween) for 40 minutes at room temperature and then incubated with a 1:2000 dilution of primary antibody in 1% milk in TBST overnight at 4°C. After washing with TBST three times, the membrane was probed with 1:2000 dilution of anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, San Francisco, CA) for 30 minutes at room temperature. Washing with TBST three times again, the membrane was developed by enhanced chemiluminescence(ECL; Amersham, Arlington Heights, IL).

**Immunoprecipitation.** Cells were washed twice with ice-cold PBS and then lysed with lysis buffer for 20 min at 4°C, and the protein content of the supernatants was determined by the bicinchoninic acid method (Pierce, Rockford, IL). Five hundred micrograms of protein was immunoprecipitated at 4°C overnight using the KDR or EGFR antibody (10 µg) and 40 µl of agarose-protein G (Roche Molecular Biochemicals). After being washed twice with lysis buffer, the KDR or EGFR immune complex was resolved by SDS-PAGE and immunoblot assay was performed as described above.

**Phosphorylation Assays.** Cells were seeded in 24-well-plate in 10% FBS RMPI1640 medium. Next day the cells were washed twice with serum-free RMPI1640 medium, and starved the cells overnight (14 hours). On the third day, cells were treated by different

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concentrations of compounds for 30 minutes at 37 °C before the cells stimulated by VEGF for 5 min. Then cell lysates were collected. Immunoprecipitation was conducted using anti-KDR or anti-EGFR antibodies, KDR, EGFR, phosphorylated KDR or phosphorylated EGFR were examined using anti-PTY or anti-KDR or anti-EGFR antibodies through immunoblot analysis shown in above.

**Reversibility Test Protocol.** We have used the protocol described by Smaill *et al*(Smaill et al., 1999). Treated cells were washed with warm serum-free media, incubated for 30 min, washed again and incubated another 8 h. This set of cells was tested for phosphorylation.

**Cell Viability Assay.** Briefly, cells were seeded in 96-well-plate (Falcon, Lincon Park, NJ) at the various density per well. On the next day, compound was prepared at 2-fold dilutions in medium and incubated with the cells till the cells of control grew 100% confluent. Cell viability was determined by MTT assay. Briefly, MTT (Sigma M-2128) was dissolved and sterilized in PBS at 5 mg/ml and 10 μl was added into each well. The plate was incubated in incubator (37°C, 5% $CO_2$) for 4 hours and all the medium was removed. Add DMSO 100 μl into each well to dissolve the dark blue crystal, shake the plate gently 5 minutes. Read OD values with a test wavelength of 570 nm and a reference wavelength of 650 nm(Zhu et al., 2003).

**In vivo Antitumor Activity.** The human liver and lung cancer models were established in nude mice. BALB/c nude mice were obtained from Experimental Animal Center, Sun Yat-sen University. Female mice were 4-6 weeks old. All manipulations were performed under sterile condition. The procedures involving mice and their care were in accord with the NIH guide for the Care and Use of Laboratory Animals and with United Kingdom Co-ordinating Committee on Cancer Research Guidelines for the Welfare of Animal in Experimental Neoplasia (Second Edition) (British Journal of Cancer 1998, 77(1):

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1-10). Tumor xenografts were established by $1\times10^6$ Bel7402 cells injected into nude mice subscutuously. Tumors were measured with a caliper in larger diameter and smaller diameter. The tumor volume was calculated by formula 1/2 ×larger diameter ×(smaller diameter)$^2$. Treatments were initiated on day 7 post inoculation, the volume reached ~20 mm$^3$ at this time. ON-III was tested alone in mice bearing transplanted Bel7402 cells and GLC-82 cells for determining the efficacy dosage for these tumors. ON-III was given at 5.0mg/kg, 10.0mg/kg and 20.0mg/kg i.p. five times a week for 4 weeks. Approximate tumor sizes and body weight were measured twice a week. Average volumes and standard derivations were calculated for each group and plotted. Finally, the mice were sacrified, the tumor tissues were excised and weighed. The growth inhibitory effect was calculated with formula: inhibitory rate (%)= (T control – T drug)/ T control ×100%.

**Quantification of Tumor Vessel Counts.** To determine whether tumor growth inhibition by ON-III was associated with inhibition of tumor vessel formation, groups of mice bearing the Bel7402 hepatocarcinoma were used for histological examination. The tumor-bearing mice were treated with either 20mg/kg/d of ON-III or vehicle. Two weeks after starting treatment, the tumor tissues was quickly frozen and stored at –70 □. Frozen sections were fixed and stained with primary antibodies to CD31. Five random 0.159 mm$^2$ fields at ×200 magnification were captured for each tumor by using a sony three-chip camera mounted on a Zeiss universal microscope(Drevs et al., 2000; Laird et al., 2002).

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## Results

**The effect of ON-III on tyrosine phosphorylation of KDR.** Autophosphorylation of KDR increases the velocity of the kinase reaction and only phosphorylated KDR can provide binding sites for its substrates (such as phospholipase Cγ, Grb-2, Shc, etc.). Thus, we firstly detected that ON-III inhibited tyrosine phosphorylation of KDR. To examine the relationship of dose-effect of inhibition, HDMEC cells were treated in serum-free medium with indicated concentrations of ON-III for 30 min, then stimulated with 10ng/ml VEGF for 5 min. Cells were lyzed and immunoprecipitated using KDR antibody, then resolved in SDS-PAGE. Western blot analysis was conducted with anti-phospho-tyrosine antibody and KDR antibody respectively. The results suggested that ON-III significantly inhibited phosphorylation of KDR under the concentrations of 0, 2.5, 5.0, 10.0, 20.0 μg/ml with dose-dependent manner, $IC_{50}$ value is 6.20 μg/ml in HDMEC cells. But the levels of KDR protein were unaffected following ON-III treatment (Fig. 2).

**The selectivity of inhibition of KDR phosphorylation by ON-III.** ON-III inhibited tyrosine phosphorylation of KDR. It is of interest to investigate the relative selectivity of ON-III toward KDR. To elucidate this issue, human epidermal EGFR-overexpressing cell line A431 cells were employed. A431 cells were treated with 10 and 40 μg/ml of ON-III for 30 min, then were lyzed and immunoprecipitated using EGFR antibody, resolved in SDS-PAGE. Western blot analysis was conducted with anti-phospho-tyrosine antibody and EGFR antibody respectively. The results showed that ON-III had no inhibitory effect on tyrosine phosphorylation of EGFR following over 40 μg/ml of ON-III treatment for 30 min in A431 cells (Fig. 3).

**Reversible inhibition of KDR tyrosine kinase phosphorylation by ON-III.** The results shown above indicate that ON-III selectively inhibits tyrosine phosphorylation of KDR receptor. To investigate whether inhibition of tyrosine phosphorylation of KDR by ON-III is

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reversible, HDMEC cells were treated with 10μg/ml ON-III in serum-free medium for 30 min and the cells were washed with serum-free medium twice, then incubated for 30 min and 8 h separately. The tyrosine phosphorylation of KDR was detected. The results showed that the levels of phosphorylation of KDR completely recovered 8 h after ON-III was washed out (Fig. 4).

**Effect of ON-III on the activation of MAPK and AKT, downstream targets of KDR signal pathway.** Activation of KDR leads to endothelial cell proliferation, presumably by inducing activation of extracellular signal regulated kinase 1, 2 (Erk1, 2) and PI3K/AKT pathway. To examine whether ON-III can inhibit activation of MAPK and AKT, HDMEC cells were treated with varying concentrations of ON-III for 30min, phospho-MAPK and MAPK was detected with anti-phospho-MAPK and anti-MAPK antibody in HDMEC cells. Phospho-Akt and Akt were detected with anti-phospho-AKT and anti-AKT antibody. Phospho-MAPK and phospho-Akt are the active type of MAPK and Akt respectively. The results showed that phospho-MAPK and phospho-Akt decreased significantly after treatment of HDMEC cells with ON-III (Fig. 5), but total MAPK and Akt were unaffected. However, phospho-MAPK and phospho-AKT in A431 were unaffected by ON-III (data no shown). It suggested that ON-III has potent inhibitory effect on activation of MAPK and Akt in a dose-dependent manner in VEGF-stimulating HDMEC cells following treatment with ON-III for 30 min.

**Effect of ON-III on the growth of human endothelial cells in the presence of VEGF.** The results described above suggested that ON-III inhibited tyrosine phosphorylation of KDR in HDMEC cells. It should be noted whether ON-III could inhibit cell growth more potent in the presence of VEGF. In order to elucidate this issue, HDMEC cells and MTT analysis were employed. The results shown in Fig. 6 indicated that ON-III had more potential inhibitory effect on HDMEC cells in the presence of VEGF. It indicated that ON-III inhibited

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VEGF-mediated cell growth in HDMEC cells.

**Antitumor activity of ON-III in vivo.** ON-III inhibited KDR tyrosine kinase phosphorylation and KDR-mediated signal transduction. It suggested that ON-III might inhibit the tumor growth in vivo. So we established human liver cancer and lung adenocarcinoma xenografts to detect antitumor activity of ON-III in vivo. ON-III administration was initiated on day 7 post implantation, when the tumor size was approximately ~20 $mm^3$. ON-III was alone given by i.p. every day at dose of 5, 10, 20mg/kg and tumor growth was inhibited with 38%, 45% and 65% respectively after four weeks treatment, there are significant difference ($P<0.05$) compared to control )(Fig. 7a). No obvious toxicity was observed in mice receiving the dosage treatment. ON-III had no effect on body weight of nude mice bearing hepatocarcinoma cell Bel7402 (Table 1). Also, ON-III inhibited human lung cancer GLC-82 xenografts in nude mice. The inhibitory rates were 21.8% ($P>0.05$), 40% ($P<0.01$) respectively after four weeks treatment at dose of 5, 20 mg/kg of ON-III (Fig. 7b).

**Effect of ON-III on tumor vascularization.** ON-III can inhibit VEGFR tyrosine kinase phosphorylation. Thus, inhibition of tumor growth in vivo by ON-III may be through inhibiting tumor angiogenesis. Therefore, we employed immunohistochemical staining for CD31 to detect vessels in tumor tissues. The results revealed a significant decrease in tumor vessel counts in the ON-III groups compared with those in the control group (Fig. 8).

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## Discussion

Tumor growth depends on angiogenesis. This angiogenic switch is characterized by oncogene-driven tumor expression of pro-angiogenic proteins, such as VEGF, basic fibroblast growth factor (bFGF), interleukin-8 and others. The significance of VEGF/VEGFR signaling in tumor initiation, progression and metastasis make them important targets for development of specific inhibitors(Blagosklonny, 2004). Activation of KDR by VEGF results in its receptor tyrosine kinase phosphorylation. Phosphorylated KDR receptor provides binding sites for SH-2 domain-containing protein including the adaptor protein Grb-2 and Shc. In addition to SH-2 domain, Grb-2 binds to the small nucleotide exchange proteins such as Sos through its SH-3 domain, then activating ras through exchanging GTP for GDP on ras(Jeong et al., 2002). Activated ras binds to and facilates raf activation. Activated raf stimulates MEKK activity which, in turn, activates the MAPK pathway. Inhibiting VEGF production and blocking VEGF/VEGFR signaling can inhibit tumor angiogenesis and tumor growth in vivo (Keyes et al., 2004; Teicher et al., 2001). Although some small molecules targeting KDR tyrosine kinase e.g. SU5416, SU6668, ZD6474 and PTK787 had been reported, it is very interesting to identify VEGFR tyrosine kinase inhibitors from Traditional Chinese Medicine (TCM) because TCM has usually no or low toxicity to host (Carlomagno et al., 2002; Mendel et al., 2003; O'Farrell et al., 2003).

KDR tyrosine kinase is a potent target for cancer treatment (Huang et al., 2002; Itokawa et al., 2002). In this study, we found that ON-III, which was one of the chalcone derivatives from *Cleistocaly xoperculatus* dry flower, inhibited tyrosine phosphorylation of KDR. But the expression of KDR protein was unaffected. It indicates that inhibition of phosphorylation of KDR was not via reduced KDR expression. The data demonstrated that inhibition of KDR phosphorylation by ON-III was reversible and relatively selective compared to EGFR tyrosine kinase phosphorylation. Our results demonstrated that ON-III inhibited activation of MAPK

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and Akt and shut down KDR downstream signaling. ON-III inhibited VEGF-stimulating HDMEC cell proliferation significantly. In comparison, growth inhibition of HDMEC cells by ON-III in the presence of EGF, but without VEGF, was obviously weaker. Moreover, ON-III produced a dose-dependent inhibition of tumor growth in human tumor xenograft models, hepatocarcinoma Bel7402 and lung adenocarcinoma GLC-82, despite their varied histological origin (liver, lung) and different growth inhibitory rates. In both cases, the percentage of inhibition on tumor growth keep increasing as the duration of drug application was extended, which is indicative of a sustained antitumor effect of ON-III. Furthermore, histological examination showed that tumor vessel counts decreased in tumor tissues in ON-III-treated mice. It may be the result of VEGF signaling blockage. In all of the in vivo experiments described in this report, ON-III was extremely well tolerated with no obvious effects on body weight or animal behavior, and no target organ toxicity was observed.

From this experiment, we found that efficacy of ON-III after daily i.p. dosing was dependent on the growth rate of the tumors and was more optimal against slower-growing tumors and more variable against fast-growing tumors. Our findings that ON-III is less effective against lung carcinoma GLC-82 than hepatocarcinoma cancer Bel7402 is consistent with studies reported using the tyrosine kinase inhibitors, SU5416 and PTK787/ZK222584, and neutralizing antibodies against VEGF(Laird et al., 2000; Mendel et al., 2000). These inhibitors are also less effective against some tumors than others. This effect may be due to differences in the requirement of new blood vessels for the growth of particular tumors and the angiogenic factors produced by a particular tumor cell population. Although others have shown that almost all tumor cell lines in vitro and tumors grown in vivo produce VEGF, cytokines or growth factors other than VEGF may also contribute to endothelial cell survival and tumor angiogenesis and even be up-regulated when the effects of VEGF are inhibited (Buchdunger et al., 2000; Fong et al., 1999). In vitro we have observed that ON-III had low

inhibitory effect on endothelial cell growth if EGF is present in the medium. This suggests that there are several different factors supporting endothelial survival and tumor vascularization. Anti-VEGF therapy may be more effective against some types of tumors than others, and future therapy may necessitate a combination of antiangiogenic agents with different mechanisms of action, as well as conventional therapies targeting the tumor cells. This may give VEGF inhibitors additional therapeutic applications over antiangiogenic agents with other mechanisms of action. Soluble VEGF receptors and antibodies against VEGF or its receptors have also been proposed as agents to block VEGF, but compared with ON-III, the antibodies have the disadvantages of large proteins (Dias et al., 2001; Klement et al., 2000).

Taken together, we identified ON-III, a potent inhibitor of VEGF signaling with antitumor effect in vivo. This compound is from Traditional Chinese Medicine *Cleistocaly xoperculatus* dry flower, which is used in China for treatment of inflammation for many years. Clinically, it has no observed adverse events. Therefore, ON-III is a promising anticancer agent targeting VEGFR tyrosine kinase.

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**Footnotes:**

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## Legends for Figures

Fig. 1. The structure of ON-III.

Fig. 2. Inhibition of KDR phosphorylation by ON-III in HDMEC cells. HDMEC cells were treated with different concentrations of ON-III for 30min. Cells were collected, lysed and immunoprecipitated using KDR antibody, then resolved in SDS-PAGE. Western blot analysis was conducted and probed with anti-KDR or anti-PTY antibody. Lower panel shows the ratios of protein quantitation of phospho-KDR to KDR. This experiment is representive of three experiments.

Fig. 3. Inhibition of EGFR phosphorylation by ON-III. A431 cells were treated with 0 (lane 1)，0.2% DMSO (lane 2), ON-III of 10 μg/mL (lane 3), 40 μg/mL (lane 4) for 30min. Cells were collected, lyzed and immunoprecipitated using EGFR antibody, then resolved in SDS-PAGE. Western blot analysis was conducted and probed with anti-EGFR or anti-PTY antibody. This experiment is representive of two experiments.

Fig. 4. Reversible inhibition of KDR phosphorylation by ON-III. HDMEC cells were treated with 0 (lane 1), vehicle (lane 2), 10μg/ml ON-III for 30min (lane 3), then were washed and incubated for 30min (lane 4) or 8h (5) with fresh medium. Immunoprecipitation was performed with KDR antibody. Western blot analysis was performed and probed with anti-PTY or anti-KDR antibody. This experiment is repeated three times.

Fig. 5. Inhibition of MAPK and AKT activation by ON-III. HDMEC cells were treated with different concentrations of ON-III for 30min in the presence of VEGF. Cells were collected and lyzed. Western blot analysis was conducted and probed with anti-phospho-MAPK, anti-

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MAPK antibody, anti-phospho-AKT or anti-AKT antibody. This experiment is representive of three experiments.

Fig. 6. Inhibitory effect of ON-III on the growth of HDMEC cells in the presence of VEGF or EGF. HDMEC cells were treated with different concentrations of ON-III in the presence of VEGF or EGF for 72 h. Cell viability was determined with MTT assay and shown as cell viability rate ± SE (n=4).

Fig. 7. The inhibitory effect of ON-III on the growth of human hepatocarcinoma cell Bel7402 (A) and lung adenocarcinoma GLC-82 (B) xenograft tumors. Bel7402 and GCL-82 cells were implanted s.c. in athymic mice. Treatment was started when palpable primary tumors reached a size of ~0.16cm$^3$. Once-daily i.p. administration of ON-III or vehicle was then started and continued for the duration of the experiment. Data points represent a mean tumor volume ± SE from 10 mice. A, ON-III has significant inhibitory effect on hepatocarcinoma cell growth with inhibition rates ranging from 38%, 45% to 65% at dosages 5, 10 or 20mg/kg respectively ($P<0.05$). B, ON-III inhibited lung cancer cell growth with inhibition rates ranging from 21.8% ($P>0.05$) to 40% ($P<0.05$) at dosages 5 or 20 mg/kg respectively.

Fig. 8. Analysis of microvessel density of ON-III-treated Bel7402 tumor xenografts (n=5). A, histological sections of control (vehicle-treated ) and ON-III (20mg/kg/day)-treated Bel7402 tumors stained for CD31 and presented at 200× magnification. A significant reduction in CD31 (endothelial cells) staining was evident after ON-III treatment. Mean CD31-positive area/5000 μ m$^2$ viable tumor (B), as determined by morphometric image analysis after ON-III treatment, are shown. $P<0.01$ by one-tailed *t* test.

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Table 1. Effect of ON-III on body weight of nude mice bearing hepatocarcinoma cell BEL7402

| Groups | before treatment | | post treatment | |
|---|---|---|---|---|
| | No. of mice | body weight (g) | No. of mice | body weight (g) |
| NS | 10 | 23.1±1.84 | 10 | 28.1±2.46 |
| DMSO | 10 | 23.4±1.93 | 10 | 26.3±1.67 |
| ON-III(5mg/kg) | 10 | 22.8±1.35 | 10 | 26.2±1.39 |
| ON-III(10mg/kg) | 10 | 23.1±1.69 | 10 | 26.9±2.52 |
| ON-III(20mg/kg) | 10 | 23.6±2.61 | 10 | 24.4±2.33 |

The treatment of ON-III has no significant effect on body weight of nude mice compared with DMSO group, $P>0.05$.

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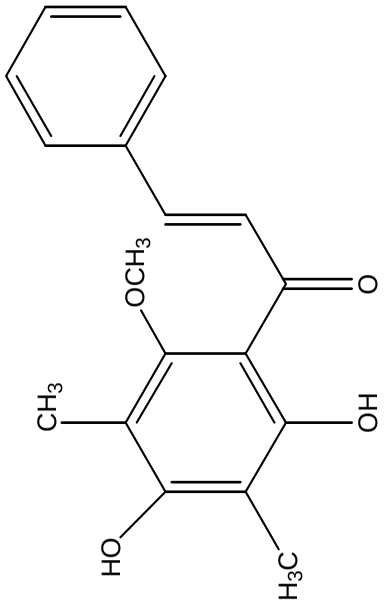
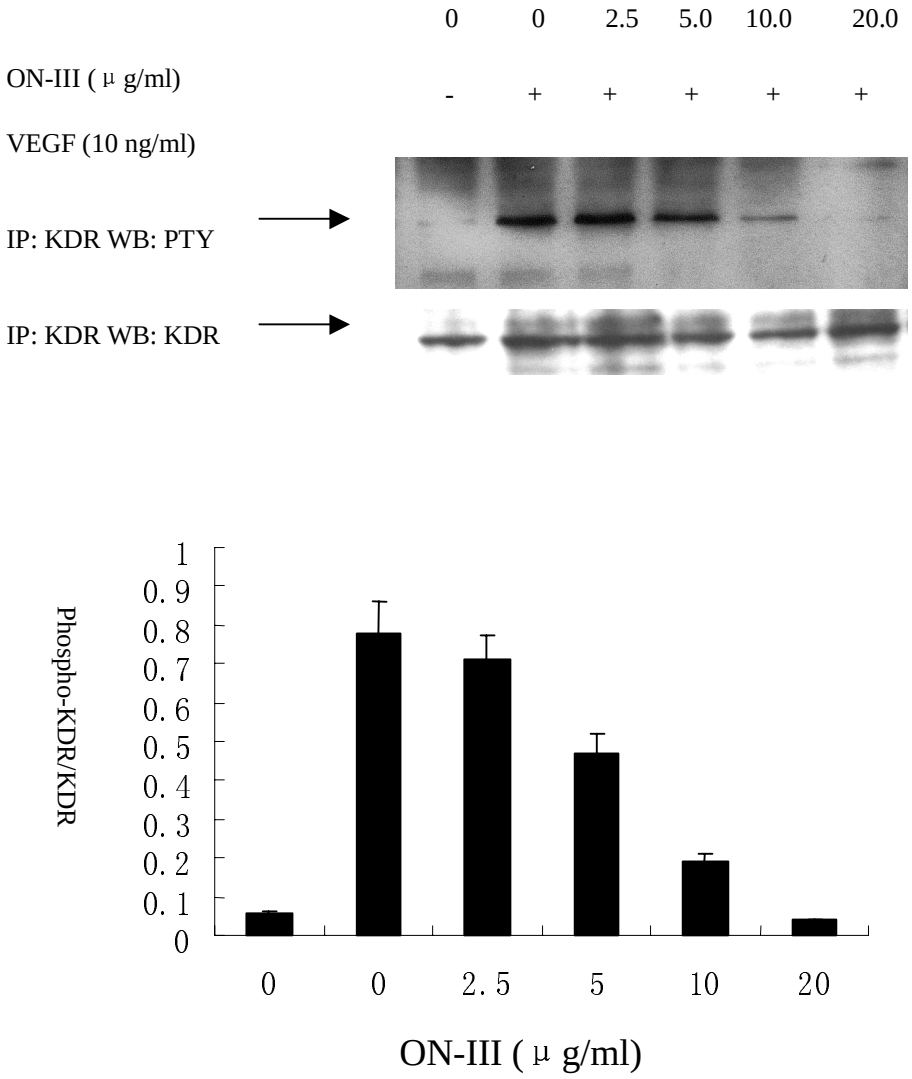



Fig 1

# Fig 2



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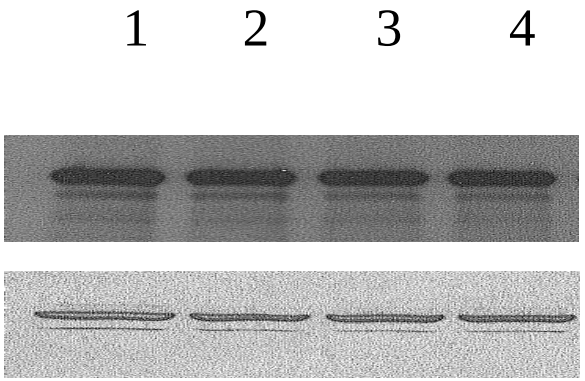

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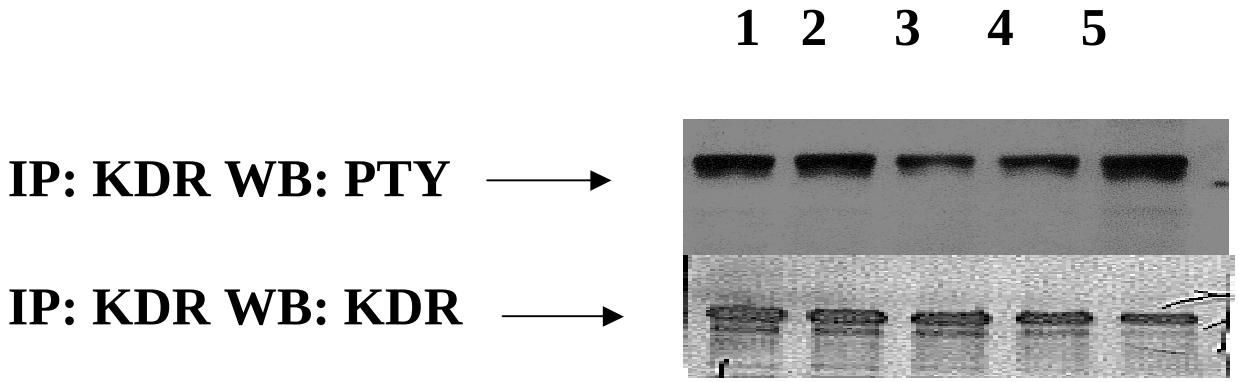
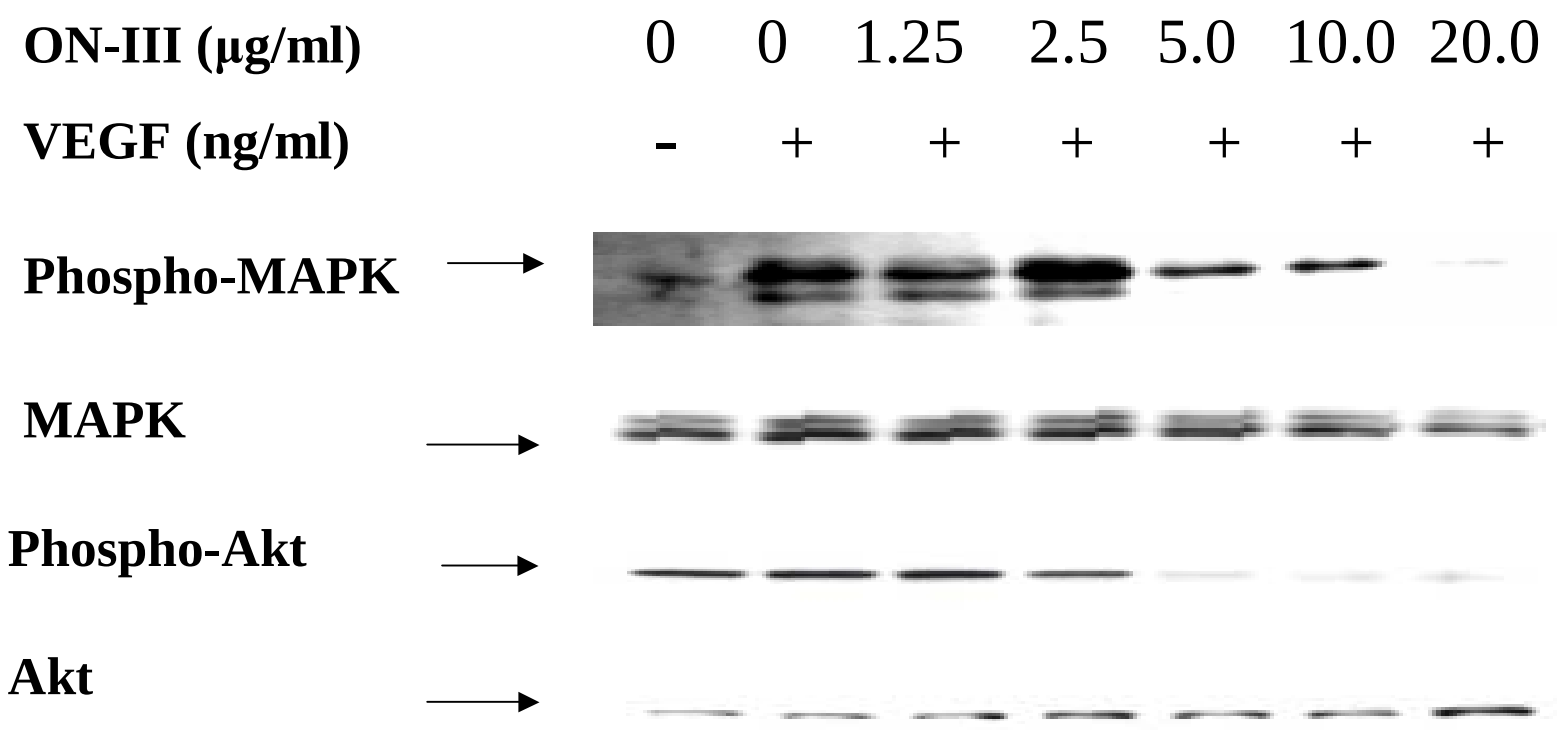


Fig 5



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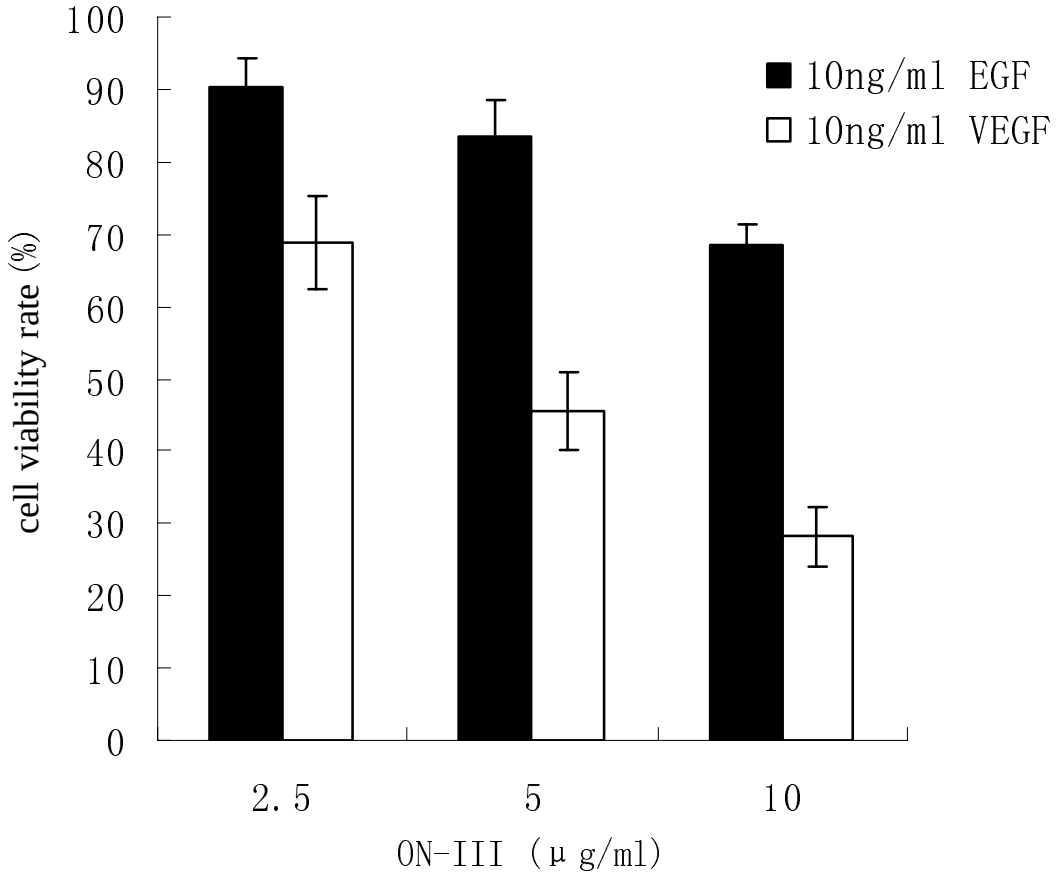
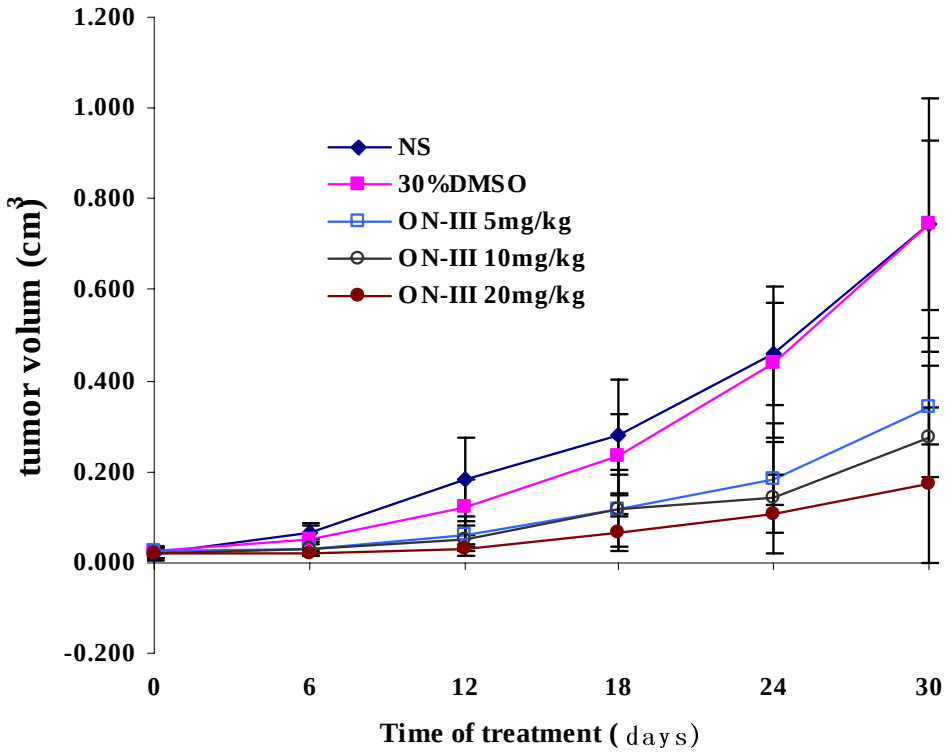


Fig 7

A.



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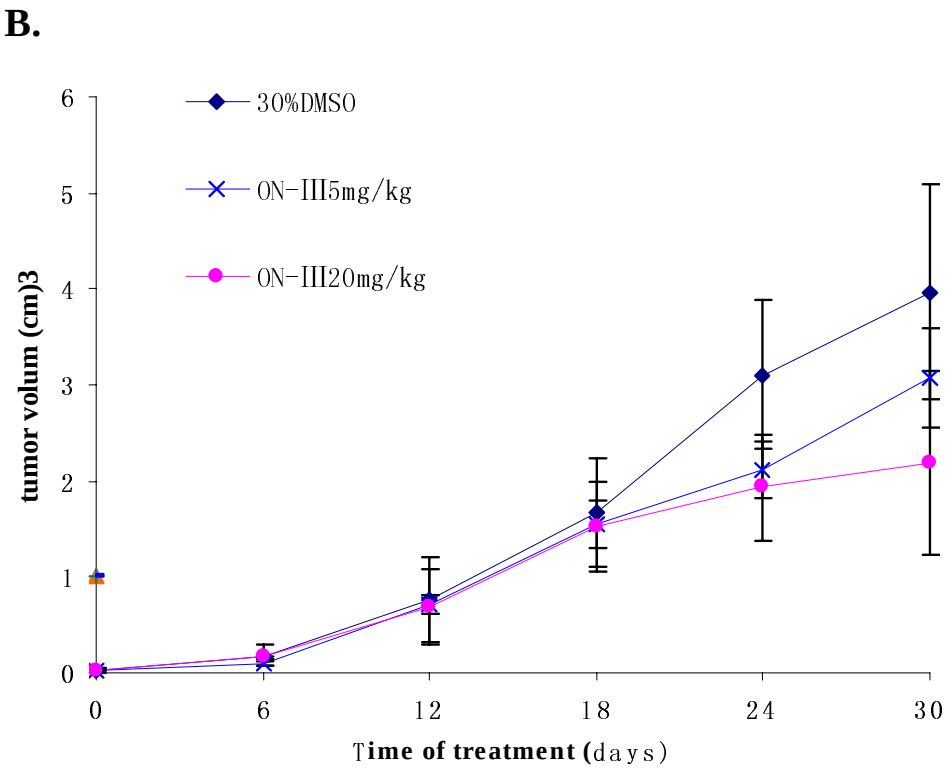

# Fig 8