# 11,11′-Dideoxy-verticillin: a natural compound possessing growth factor receptor tyrosine kinase-inhibitory effect with anti-tumor activity

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**11,11′-Dideoxy-verticillin, a compound of the novel epidithiodioxopiprazine structural class, is isolated from the traditional Chinese medicinal herb *Shiraia bambusicola*. The present study demonstrated for the first time that 11,11′-dideoxy-verticillin has potent tyrosine kinase-inhibitory and anti-tumor activities. In the cell-free ELISA tyrosine kinase assay, 11,11′-dideoxy-verticillin significantly inhibited the activities of epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor-1/fms-like tyrosine kinase-1 (VEGFR-1/Flt-1) and human epidermal growth factor receptor-2 (HER2/ErbB-2), with relative specificity on EGFR and VEGFR-1 with $IC_{50}$s of 0.136 ± 0.109 and 1.645 ± 0.885 nM, respectively. Exposure of 11,11′-dideoxy-verticillin for 1 h to EGFR-overexpressed MDA-MB-468 human breast carcinoma cells and HER2-overexpressed SK-OV-3 human ovarian adenocarcinoma cells resulted in obvious inhibition of EGF-induced phosphorylation of EGFR and HER2. In addition, 11,11′-dideoxy-verticillin also inhibited the EGF-induced phosphorylation of Erk1/2, but had no effect on the phosphorylation of AKT in both tumor cell lines. Moreover, 11,11′-dideoxy-verticillin has potent anti-tumor activity. *In vitro* cytotoxicity assay showed that 11,11′-dideoxy-verticillin potently inhibited the proliferation of four human breast tumor cell lines with an average $IC_{50}$ value of 0.2 μM. *In vivo*, 11,11′-dideoxy-verticillin exhibited remarkable efficacy against mice sarcoma 180 and hepatoma 22 after daily i.p. administration of 0.5 or 0.75 mg/kg with inhibition rates ranging from 45.0 to 72.4%. Treated with 11,11′-dideoxy-verticillin at 0.5–2.0 μM for 36 h, MB-MB-468 cells exhibited significant apoptotic morphological changes. At low concentrations (0.0625–0.5 μM) for 24 h, 11,11′-dideoxy-verticillin induced a dose-dependent accumulation of MDA-MB-468 cells in the $G_2$/M phase of the cell cycle. These results indicate that 11,11′-dideoxy-verticillin is a naturally derived growth factor receptor tyrosine kinase inhibitor with potent anti-tumor activity. *Anti-Cancer Drugs* 16:515–524 © 2005 Lippincott Williams & Wilkins.**

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## Introduction

Receptor tyrosine kinases (RTKs), which initiate specific intracellular signaling pathways in response to binding of extracellular growth factors, play an important role in regulating a wide variety of cellular functions, including regulation of mitogenesis, cell death and cell differentiation. Deregulated expression of RTKs is responsible for tumor development and progression, including the promotion of proliferation, angiogenesis, invasion/metastasis and chemotherapy resistance [1–3]. Thus, members of this RTK family have been identified as attractive target candidates for targeted cancer therapy. Over the past few years, tremendous progress has been made with respect to the preclinical and clinical development of RTK inhibitors, which is reflected in the fact that several RTK inhibitors are now in the advanced stages of clinical trials or have already been approved as therapeutic agents to treat a variety of cancers [4–6]. The most convincing evidence is the epidermal growth factor receptor (EGFR) inhibitor Iressa being launched in Japan and recently in the US for use in refractory non-small cell lung cancer. Unfortunately, most patients with non-small cell lung cancer have no response to Iressa [7,8]. Even in gliomas, despite the fact that the high frequency of amplification and rearrangements of the EGFR gene suggested that EGFR plays an important role, Iressa failed to drive clinically significant responses simply due to its limited therapeutic value [9,10]. Current views hold that

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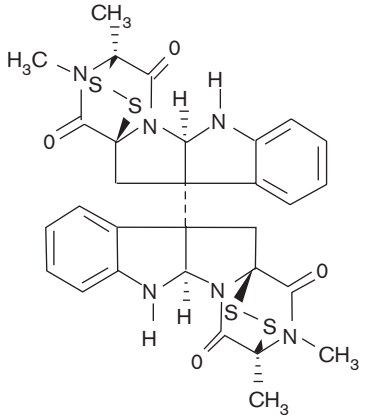
combined blockage on both EGFR and vascular endothelial growth factor receptor (VEGFR) functions or more broadly non-specific inhibitors might lead to beneficial clinical effects [11]. This approach has become a center of focus for the development of potential therapeutic agents against cancers. In this respect, efforts strive to identify non-specific RTK inhibitors that specifically arrest both EGF and VEGF receptor activities or more within the same molecule.

Natural products are rich resources of chemical diversity and potential therapeutic leads. Of them, traditional Chinese medicine has attracted tremendous interest due to its considerable yield of new anti-tumor chemical entities. In the present study, we preferentially aimed at finding potential RTK inhibitors as anti-cancer drug candidates from traditional Chinese medicinal herbs based on their use in folk medicine. *Shiraia bambusicola*, a fungus that inhabits bamboo, is mainly distributed in several provinces in south China. In Chinese folk medicine, *S. bambusicola* was used to treat psoriasis. Psoriasis is a heterogeneous skin disease, characterized by epidermal hyperproliferation, abnormal keratinization and inflammation. Importantly, the overexpression of EGFR is the hallmark of psoriasis [12]. Encouragingly, AG 1517 (SU 5271), a potent EGFR inhibitor, is currently in advanced clinical trials for the treatment of psoriasis [13]. It was for this reason that we began to isolate and identify the bioactive components from *S. bambusicola*. In this report, 11,11′-dideoxy-verticillin is established (Fig. 1) to be a small molecule compound isolated from *S. bambusicola*. We assessed the inhibitory effects of this compound on EGFR, human epidermal growth factor receptor 2 (HER2/ErbB-2) and VEGFR-1/Flt-1, and consequently tested its anti-tumor activity *in vitro* and *in vivo*.

**Fig. 1**



Chemical structure of 11,11′-dideoxy-verticillin.

## Material and methods

### Compounds

11,11′-Dideoxy-verticillin presenting as a white-colored crystalloid with purity 99% was isolated from the fungus *S. bambusicola* by the Phytochemistry Department of Shanghai Institute of Materia Medica. *S. bambusicola Henn* was powdered and refluxed with petroleum ether (5 l × 3) for 1 h. The defatted residue was then refluxed with 95% ethanol (5 l × 2) for 2 h. The combined ethanol extract was evaporated *in vacuo* to give 400 ml of aqueous residue, which was further partitioned with chloroform (400 ml × 3). The chloroform extract was subjected to column chromatography over silica gel with a petroleum ether–acetone gradient (4:1 → 1:1) as eluent to yield 11,11′-dideoxy-verticillin (10 mg). PD153035, as a positive control of EGFR inhibition, was obtained from Calbiochem (Darmstadt, Germany) [14]. AG825, a known ErbB-2 tyrosine kinase inhibitor, was obtained from Sigma-Aldrich (St Louis, MO) [15]. Compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) as stock solution. Stock solutions were stored in aliquots at –20°C and thawed just before the test. The concentration of DMSO was below 0.1% (v/v) in 11,11′-dideoxy-verticillin-treated groups. DMSO 0.1% (v/v) was used as a vehicle control throughout the study.

### Cell line and culture conditions

Human breast adenocarcinoma MDA-MB-468, MCF-7, MDA-MB-435 and MDA-MB-231 cells and the human ovarian adenocarcinoma SK-OV-3 were obtained from ATTC (Manassas, VA). Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 kIU/l benzylpenicillin, 100 mg/l streptomycin and 10 mM HEPES (pH 7.4) in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C.

### Tyrosine kinase assay

For kinase assays, purified EGFR was purchased from Sigma. The GST-fusion proteins of the kinase domain of VEGFR-1/Flt-1 (amino acids 812–1158) were produced in an *Escherichia coli* expression system as previously described [16] and purified by affinity chromatography on a glutathione–Sepharose 4B column (Amersham Life Sciences, Arlington Heights, IL). The kinase domain of human HER2/ErbB-2 (amino acids 676–1091) was expressed using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA) and purified by a Ni-NTA column (Qiagen, Valencia, CA).

The tyrosine kinase activities of the purified EGFR, GST-VEGFR-1 and HER2 were determined on the 96-well format ELISA models. Briefly, 40 µl of 5 µM ATP solution diluted in kinase reaction buffer solution (50 mM HEPES, pH7.4, 20 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.2 mM $Na_3VO_4$ and 1 mM DTT) was added to 96-well microtiter plates precoated with 20 µg/ml

poly(Glu,Tyr)$_{4:1}$ (Sigma). Then, 10 µl of diluted compounds was added to each reaction well to produce a range of inhibitor concentrations appropriated for each enzyme. Subsequently the kinase reaction was initiated by the addition of 0.25 U EGFR, 0.5 µg GST-VEGFR-1 or 0.78 µg HER2 diluted in kinase reaction buffer solution. After incubation for 60 min at 37°C, the plate was washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20. Then, 100 µl mouse monoclonal anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 400 ng/ml in PBS containing 0.1% Tween 20/5 mg/ml bovine serum albumin (BSA) was added to the plate and incubated at room temperature for 60 min. After washing with PBS containing 0.1% Tween 20, 100 µl of goat anti-mouse IgG conjugated to horseradish peroxidase (Calbiochem) was added to the plate at a 1/2000 dilution in PBS containing 0.1% Tween 20/5 mg/ml BSA. The plate was incubated at room temperature for 60 min and washed as before with PBS containing 0.1% Tween 20. Finally, 100 µl of a solution (0.03% $H_2O_2$, 2 mg/ml *o*-phenylenediamine in citrate buffer 0.1 M, pH 5.5) was added and incubated at room temperature until color developed. The reaction was stopped by the addition of 50 µl of 2 M $H_2SO_4$ and the plates were measured using a multi-well spectrophotometer (VERSAmax; Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm. The rate of inhibition of tyrosine kinase activity was calculated using the following formula: inhibition rate = $[1 - (A_{490\ \text{treated}}/A_{490\ \text{control}})] \times 100\%$.

The results were expressed as $IC_{50}$ values, which were defined as the drug concentration that resulted in 50% inhibition of enzyme activity.

#### Western blot analysis

MDA-MB-468 or SK-OV-3 cells were grown to 50% confluency in six-well plates and incubated in serum-free medium for 18 h. Cells were then exposed to 11,11′-dideoxy-verticillin at gradient concentrations for 1 h before 20 ng/ml EGF (R & D Systems, Minneapolis, MN) stimulation for 10 min at 37°C. After rinsing with ice-cold PBS containing 1 mM $Na_3VO_4$, cells were lysed in 0.1 ml of Laemmli buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 1 mM $Na_3VO_4$ and 0.1% bromophenol blue). Cell lysates were analyzed on 7.5% SDS–PAGE and transferred to nitrocellulose membranes (Amersham Life Sciences). After transfer, blots were incubated with the blocking solution and probed with rabbit polyclonal anti-phospho-EGFR antibody, rabbit polyclonal anti-phospho-HER2 antibody, rabbit polyclonal anti-phospho-ERK1/2 antibody and rabbit polyclonal anti-phospho-AKT antibody (all from Cell Signaling Technology, Beverly, MA), followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Calbiochem). Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (Pierce, Rockford, IL). To determined total EGFR, HER2, ERK1/2 and AKT levels, membranes were stripped with Reblot solution (Chemicon, Temecula, CA), and reprobed with rabbit polyclonal anti-EGFR antibody, rabbit polyclonal anti-HER2 antibody, rabbit polyclonal anti-ERK1/2 antibody and rabbit polyclonal anti-AKT antibody (all from Cell Signaling Technology), followed by detection using a similar procedure to that described above.

#### Cytotoxicity assays

The growth inhibitory effect of 11,11′-dideoxy-verticillin on four human breast adenocarcinoma cell lines was measured by the sulforhodamine B (SRB; Sigma) assay [17]. Briefly, cells were plated in 96-well plates ($5 \times 10^3$ cells/90 µl/well) and cultured at 37°C for 24 h. Then, 10 µl of each serial dilution of 11,11′-dideoxy-verticillin in medium containing 0.1% DMSO was added to each well and the plates were incubated at 37°C for 72 h. The cells were fixed by gentle addition of 100 µl cold (4°C) 10% trichloroacetic acid to each well, followed by incubation at 4°C for 1 h. The plates were washed 5 times with deionized water, allowed to air dry and stained by the addition of 100 µl SRB solution [0.4% SRB (w/v) in 1% acetic acid (v/v)] to each well for 15 min. The plates were washed 5 times with 1% acetic acid to remove unbound dye and allowed to air dry. The bound dye in each well was dissolved in 10 mM Tris base (pH 10.5) and the OD at 515 nm was measured with a multi-well spectrophotometer (VERSAmax; Molecular Devices). The inhibition of proliferation was calculated as $[1 - (A_{515\ \text{treated}}/A_{515\ \text{control}})] \times 100\%$. The result was also expressed as $IC_{50}$ (i.e. the drug concentration that reduced the absorbance observed in untreated cells by 50%), which was calculated by the Logit method. The mean $IC_{50}$ was determined from the results of three independent tests.

#### Animals and experimental procedures for *in vivo* tests

Kunming strain female mice (certificate no. 005, weighing 20 ± 2 g) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences. Sarcoma 180 and hepatoma 22 cell suspensions were implanted s.c. into the right axilla region of mice. Daily treatment with drugs or normal saline commenced 1 day after implantation of cells. Mice were administered by i.p. injection with vehicle or 11,11′-dideoxy-verticillin once daily for consecutive days. Animals were euthanized 8 days post-tumor implantation, and the tumors were excised and weighed. The rate of inhibition of tumor growth *in vivo* was calculated using the following formula: growth inhibition = (average tumor weight of control group–average tumor weight of test group)/average tumor weigh of control group × 100%.

#### Cell cycle analysis

MDA-MB-468 cells were seeded at $5 \times 10^5$ cells/well into six-well cell culture plates and allowed to attach

overnight. Cells were then exposed to 0.05% DMSO (v/v) or 11,11′-dideoxy-verticillin at concentrations of 62.5, 125, 250 or 500 nM for 24 h and harvested. After washing with ice-cold PBS (pH 7.4), cells were fixed in 70% ethanol at 4°C for at least 2 h. Fixed cells were stained in PBS containing 10 mg/l RNase and 50 mg/l propidium iodide (PI; Sigma) 50 mg/l in the dark at 25°C for 30 min. DNA content analysis was performed by flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA) with CellQuest and ModFIT LT software (Becton Dickinson).

### Determination of apoptosis

MDA-MB-468 cells were seeded at $5 \times 10^5$ cells/well into six-well cell culture plates and allowed to attach overnight. After being exposed to 0.2% DMSO (v/v) or 11,11′-dideoxy-verticillin at concentrations of 0.5, 1 or 2 μM for 36 h cells were harvested and washed with PBS, fixed in 2% paraformaldehyde (in PBS, pH 7.4) at room temperature for 1 h and then transferred to permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing with PBS, the cells were incubated in the dark at 37°C for 1 h with the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) reaction mixture containing fluorescent-dUTP and terminal deoxynucleotidyl transferase (TdT) to catalyze attachment of fluorescently labeled dUTP to the free 3′-ends of DNA strand breaks according to the manufacturer's instructions (Roche, Mannheim, Germany). The cells were then counterstained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) for 1 min to visualize total nuclear DNA. The samples were mounted on slide glasses and photographed using fluorescent microscopy with selective band-pass filters (Olympus, Tokyo).

### Data analysis

Results were expressed as means ± SD and statistical analysis was carried out using Student's *t*-test.

## Results

### Effects of 11,11′-dideoxy-verticillin on RTK activity

The effect of 11,11′-dideoxy-verticillin on RTK activity was assayed with a cell-free ELISA method. As shown in Table 1, 11,11′-dideoxy-verticillin inhibited the tyrosine kinase activity of EGFR, VEGFR-1 and ErbB-2 in a dose-dependent manner. The compound possessed a stronger and relatively specific inhibitory effect on EGFR among these three enzymes with an $IC_{50}$ of 0.136 ± 0.109 nM. As a positive control, PD153035, a potent specific EGFR inhibitor, inhibited the tyrosine kinase activity of EGFR with an $IC_{50}$ of 1.841 ± 1.336 nM under the same conditions. The inhibitory rate of 11,11′-dideoxy-verticillin was 13.5-fold higher than that of PD153035. In addition, 11,11′-dideoxy-verticillin also significantly inhibited the tyrosine kinases activity of VEGFR-1 with an $IC_{50}$ of 1.645 ± 0.885 nM, which was 12.1-fold weaker than that on EGFR. On the other hand, 11,11′-dideoxy-verticillin showed a comparatively weak inhibitory effect on EGFR-2 with an $IC_{50}$ 2.45 ± 0.56 μM. These results indicated that 11,11′-dideoxy-verticillin possessed a wide inhibitory activity on three growth factor RTKs, with stronger activities on EGFR and VEGFR-1 enzymes.

**Table 1 Effects of 11,11′-dideoxy-verticillin on RTKs activity in cell-free system**

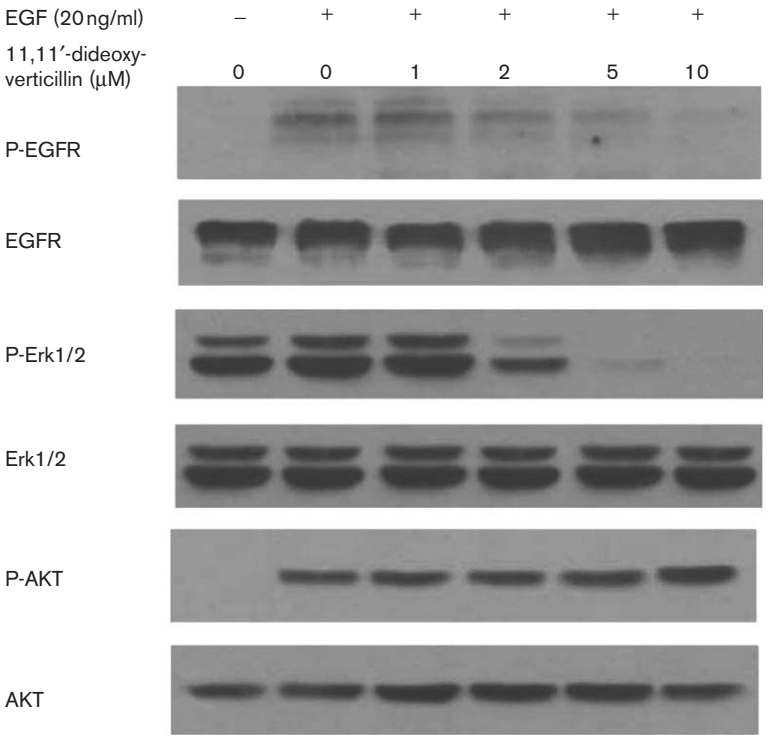
| Target and concentration (M) | Inhibition rate (%) | | |
|---|---|---|---|
| | 11,11′-dideoxy-verticillin | PD153035 | AG825 |
| EGFR | | | |
| $10^{-7}$ | 80.6 ± 18.5 | 78.5 ± 7.4 | |
| $10^{-8}$ | 75.8 ± 15.5 | 66.9 ± 8.1 | |
| $10^{-9}$ | 73.6 ± 5.7 | 43.6 ± 13.7 | |
| $10^{-10}$ | 68.0 ± 0.6 | 26.7 ± 9.5 | |
| $10^{-11}$ | 27.4 ± 7.1 | 14.0 ± 9.9 | |
| HER2 | | | |
| $10^{-4}$ | 97.4 ± 0.6 | | 94.9 ± 0.1 |
| $10^{-5}$ | 85.2 ± 5.8 | | 71.8 ± 0.3 |
| $5 \times 10^{-6}$ | 70.9 ± 9.7 | | 68.3 ± 6.6 |
| $10^{-6}$ | 21.7 ± 4.2 | | 39.9 ± 2.0 |
| $5 \times 10^{-7}$ | 17.6 ± 18.2 | | 43.8 ± 15.1 |
| $10^{-7}$ | | | 37.4 ± 11.6 |
| VEGFR-1 | | | |
| $10^{-7}$ | 81.2 ± 6.4 | | |
| $10^{-8}$ | 53.9 ± 17.0 | | |
| $10^{-9}$ | 52.0 ± 7.4 | | |
| $10^{-10}$ | 35.3 ± 11.1 | | |
| $10^{-11}$ | 17.2 ± 4.0 | | |

The ability of 11,11′-dideoxy-verticillin to inhibit the tyrosine kinase activity was directly determined by biochemical assays against the pure isolated EGFR protein, the recombinant His-HER2 protein and the recombinant GST-VEGFR-1 protein. Compounds were incubated with the enzyme and ATP in 96-well plates coated with poly(Glu,Tyr)$_{4:1}$ for 1 h and the phosphorylated tyrosine was detected by mouse monoclonal anti-phosphotyrosine antibody. The inhibition rates are shown as mean ± SD of three to six separate experiments performed in duplicate.

### Effects of 11,11′-dideoxy-verticillin on growth factor-mediated tyrosine phosphorylation in human breast carcinoma cells

To confirm the measured biochemical activity of 11,11′-dideoxy-verticillin in a cell-based assay, the effects on tyrosine phosphorylation of EGFR and HER2 after EGF stimulation were examined in EGFR-overexpressed MDA-MB-468 human breast carcinoma cells and HER2-overexpressed SK-OV-3 human ovarian adenocarcinoma cells, respectively. As shown in Figure 2, in MDA-MB-468 cells, after exposure to 11,11′-dideoxy-verticillin for 1 h, the EGF-mediated tyrosine phosphorylation of EGFR was inhibited in a dose-dependent manner. 11,11′-Dideoxy-verticillin at 2 μM inhibited the EGF-induced EGFR autophosphorylation by 50%, and at 10 μM almost completely blocked the autophosphorylation of EGFR. In SK-OV-3 cells, the EGF-mediated tyrosine phosphorylation of HER2 was inhibited in a similar dose-dependent manner (Fig. 3). 11,11′-Dideoxy-verticillin at 2 μM inhibited the EGF-induced HER2 autophosphorylation by 50% and at 10 μM inhibited the EGF-induced HER2 autophosphorylation by 90%. Overall EGFR and HER2 levels were not affected in treated cells, as evaluated in

**Fig. 2**




Effects of 11,11′-dideoxy-verticillin on EGF-induced phosphorylation of EGFR, Erk1/2 and AKT in MDA-MB-468 human breast carcinoma cells. Serum-starved MDA-MB-468 cells were treated for 1 h with the indicated concentrations of 11,11′-dideoxy-verticillin, followed by addition of EGF at a final concentration of 20 ng/ml. After 10 min at 37°C, the cells were harvested and analyzed by immunoblotting for phospho-EGFR, phospho-Erk1/2 and phospho-AKT. To determined EGFR, Erk1/2 and AKT protein levels, membranes were stripped and reprobed with antibodies against EGFR, Erk1/2 and AKT, correspondingly. Data shown are representative of three independent experiments.

parallel immunoblots using anti-EGFR antibody and anti-HER2 antibody.

### Effect of 11,11′-dideoxy-verticillin on the phosphorylation of Erk1/2 and AKT

To determine whether the inhibition of tyrosine phosphorylation of EGFR and HER2 by 11,11′-dideoxy-verticillin could affect downstream signal transduction events, the phosphorylation of Erk1/2 and AKT was detected using Western blotting. As shown in Figs 2 and 3, in MDA-MB-468 cells and SK-OV-3 cells, the phosphorylation inhibition of 11,11′-dideoxy-verticillin on Erk1/2 was similar to that seen on EGFR. In MDA-MB-468 cells, after a 1-h exposure, 11,11′-dideoxy-verticillin inhibited phosphorylation of Erk1/2 by more than 50% at 2 μM and by 100% at 10 μM. In SK-OV-3 cells, 11,11′-dideoxy-verticillin at 2 μM inhibited phosphorylation of Erk1/2 by 50% and at 10 μM inhibited the phosphorylation of Erk1/2 by 90%. Total steady-state Erk1/2 protein remained unchanged in both of these two kinds of cells. On the other hand, activation of AKT in both of MDA-MB-468 and SK-OV-3 cells was unaffected by 11,11′-dideoxy-verticillin treatment.

In addition, the effects of 11,11′-dideoxy-verticillin on EGF-mediated phosphorylation of EGFR, Erk1/2 and AKT were also confirmed in MDA-MB-231 human breast carcinoma cells. 11,11′-Dideoxy-verticillin displayed significant inhibition on phosphorylation of EGFR and Erk1/2 stimulated by EGF, but no effect on AKT phosphorylation in MDA-MB-231 cells, which was the same as that observed in MDA-MB-468 cells (data not shown).

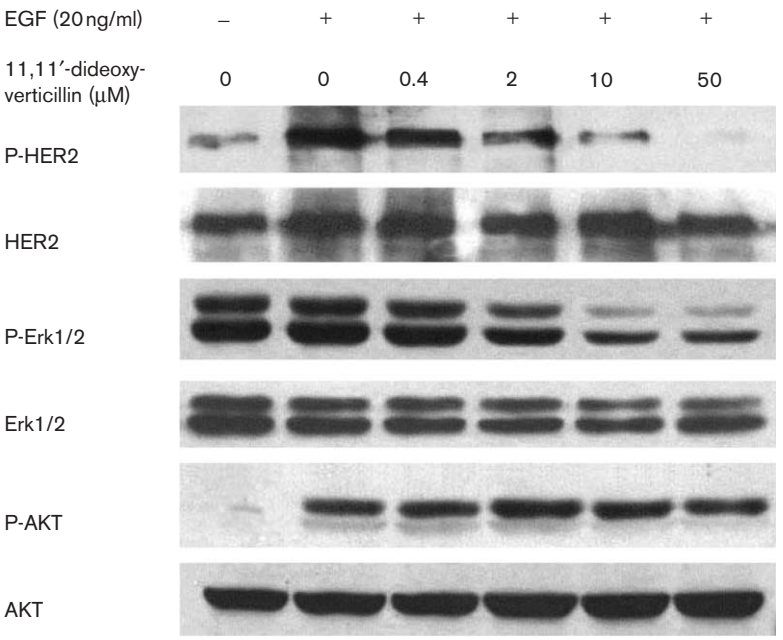
### *In vitro* cytotoxicity of 11,11′-dideoxy-verticillin

11,11′-Dideoxy-verticillin at 72-h exposure significantly decreased the proliferation of human breast cancer MDA-MB-468, MCF-7, MDA-MB-435 and MDA-MB-231 cells in a concentration-dependent manner. The $IC_{50}$ values of 11,11′-dideoxy-verticillin on MDA-MB-468, MCF-7, MDA-MB-435 and MDA-MB-231 cell lines were $0.281 \pm 0.022$, $0.158 \pm 0.070$, $0.223 \pm 0.099$ and $0.138 \pm 0.025$ μM, respectively (Fig. 4).

### *In vivo* anti-tumor effect of 11,11′-dideoxy-verticillin

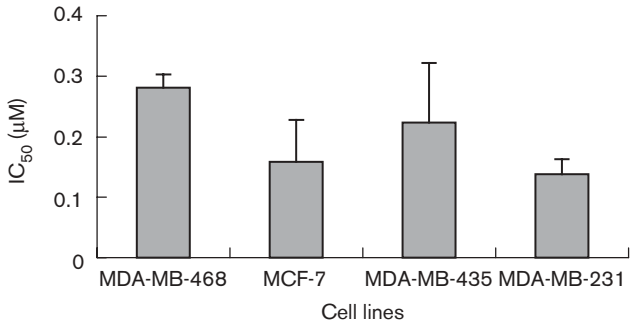
Given the potential anti-tumor effects of 11,11′-dideoxy-verticillin *in vitro*, its anti-tumor properties were determined

**Fig. 3**




Effects of 11,11′-dideoxy-verticillin on EGF-induced phosphorylation of HER2, Erk1/2 and AKT in SK-OV-3 human ovarian adenocarcinoma cells. Serum-starved SK-OV-3 cells were treated for 1 h with the indicated concentration of 11,11′-dideoxy-verticillin, followed by the addition of EGF at a final concentration of 20 ng/ml. After 10 min at 37°C, the cells were harvested and analyzed by immunoblotting for phospho-HER2, phospho-Erk1/2 and phospho-AKT. To determined HER2, Erk1/2 and AKT protein levels, membranes were stripped and reprobed with antibodies against HER2, Erk1/2 and AKT, correspondingly. Data shown are representative of three independent experiments.

**Fig. 4**




Effects of 11,11′-dideoxy-verticillin on proliferation of four human breast tumor cell lines. Cells seeded in 96-well plates were treated with various concentrations of 11,11′-dideoxy-verticillin as indicated for 72 h. Cell viability was determined by the SRB assay. The $IC_{50}$ values are shown as the mean ± SD of three independent experiments.

further *in vivo*. Table 2 presents the results of the experimental therapeutic efficacy of 11,11′-dideoxy-verticillin on mice sarcoma 180 and hepatoma 22. After daily i.p. administration with 0.5 mg/kg for 7 days, 11,11′-dideoxy-verticillin displayed significant anti-cancer efficacy ($p < 0.01$) on sarcoma 180 and hepatoma 22 tumors with an inhibition rate of 72.4 and 45.0%, respectively. When treated with 11,11′-dideoxy-verticillin at 0.75 mg/kg for 5–6 days, the inhibition rate of sarcoma 180 reached 62.7% and of hepatoma 22 reached 57.8%.

### Induction of apoptosis by 11,11′-dideoxy-verticillin

Having demonstrated significant anti-tumor effects *in vitro*, 11,11′-dideoxy-verticillin was then examined for its ability to induce apoptosis using DAPI and TUNEL staining. As shown in Fig. 5, after incubation with MDA-MB-468 human breast carcinoma cells for 36 h, 0.5–2 μM 11,11′-dideoxy-verticillin induced cell apoptosis in a dose-dependent manner as compared with the control. Characteristic nuclear morphological changes of apoptosis, including chromatin condensation, nuclear fragmentation and apoptotic bodies, were observed and became more abundant with higher concentrations of 11,11′-dideoxy-verticillin when stained by DAPI or TUNEL.

### Effects of 11,11′-dideoxy-verticillin on cell cycle distribution

The proportion of non-apoptotic MDA-MB-468 human breast carcinoma cells in the different phases of the cell cycle was determined by flow cytometry. Untreated MDA-MB-468 cells demonstrated a relatively normal distribution pattern, with most cells in the $G_0/G_1$ phase (52.02%), a lower S phase (33.48%) and $G_2$/M (14.50%) peak of the cycle. The change of cell cycle distribution of MDA-MB-468 cells treated for 24 h with gradient doses

**Table 2 Effects of i.p. administration of 11,11′-dideoxy-verticillin on the growth of sarcoma 180 and hepatoma 22 in mice**

| Tumor | Treatment group[a] | Dosage (mg/kg/day) × day | Mice (*n*) (initial/end) | Body weight (g) (initial/end) | Tumor weight (g) | Inhibition rate (%) |
|---|---|---|---|---|---|---|
| Sarcoma 180 | normal saline | – | 20/20 | 20.3/28.1 | 1.85 ± 0.52 | – |
| | 11,11′-dideoxy-verticillin | 0.5 × 7 | 10/10 | 20.1/20.4 | 0.51 ± 0.23[b] | 72.4 |
| | 11,11′-dideoxy-verticillin | 0.75 × 5 | 10/10 | 20.2/21.6 | 0.69 ± 0.24[b] | 62.7 |
| | 5-FU | 40 × 7 | 10/10 | 20.3/22.0 | 0.55 ± 0.34[b] | 70.3 |
| Hepatoma 22 | normal saline | – | 20/20 | 19.4/27.2 | 1.80 ± 0.61 | – |
| | 11,11′-dideoxy-verticillin | 0.5 × 7 | 10/10 | 19.2/21.3 | 0.99 ± 0.37[b] | 45.0 |
| | 11,11′-dideoxy-verticillin | 0.75 × 6 | 10/10 | 19.2/20.6 | 0.76 ± 0.41[b] | 57.8 |
| | 5-FU | 25 × 7 | 10/10 | 19.4/23.6 | 1.06 ± 0.39[b] | 41.1 |

[a]Sarcoma 180 cell suspensions or hepatoma 22 cell suspensions were implanted s.c. into the right axilla region of Kunming strain female mice (weighing 20 ± 2 g). Daily i.p. injections of normal saline or drugs commenced 1 day after implantation of cells. All mice were euthanized on day 8 after implantation of cells and the tumors were weighed. Data are expressed as means ± SD.
[b]$p < 0.01$ versus normal saline group.

of 11,11′-dideoxy-verticillin is shown in Table 3. Along with the increase in 11,11′-dideoxy-verticillin concentration, the proportion of $G_0/G_1$ phase cells gradually decreased, while the proportion of $G_2$/M phase cells increased. This indicates that 11,11′-dideoxy-verticillin caused the arrest of MDA-MB-468 cells in the $G_2$/M phase in a concentration-dependent manner.

## Discussion

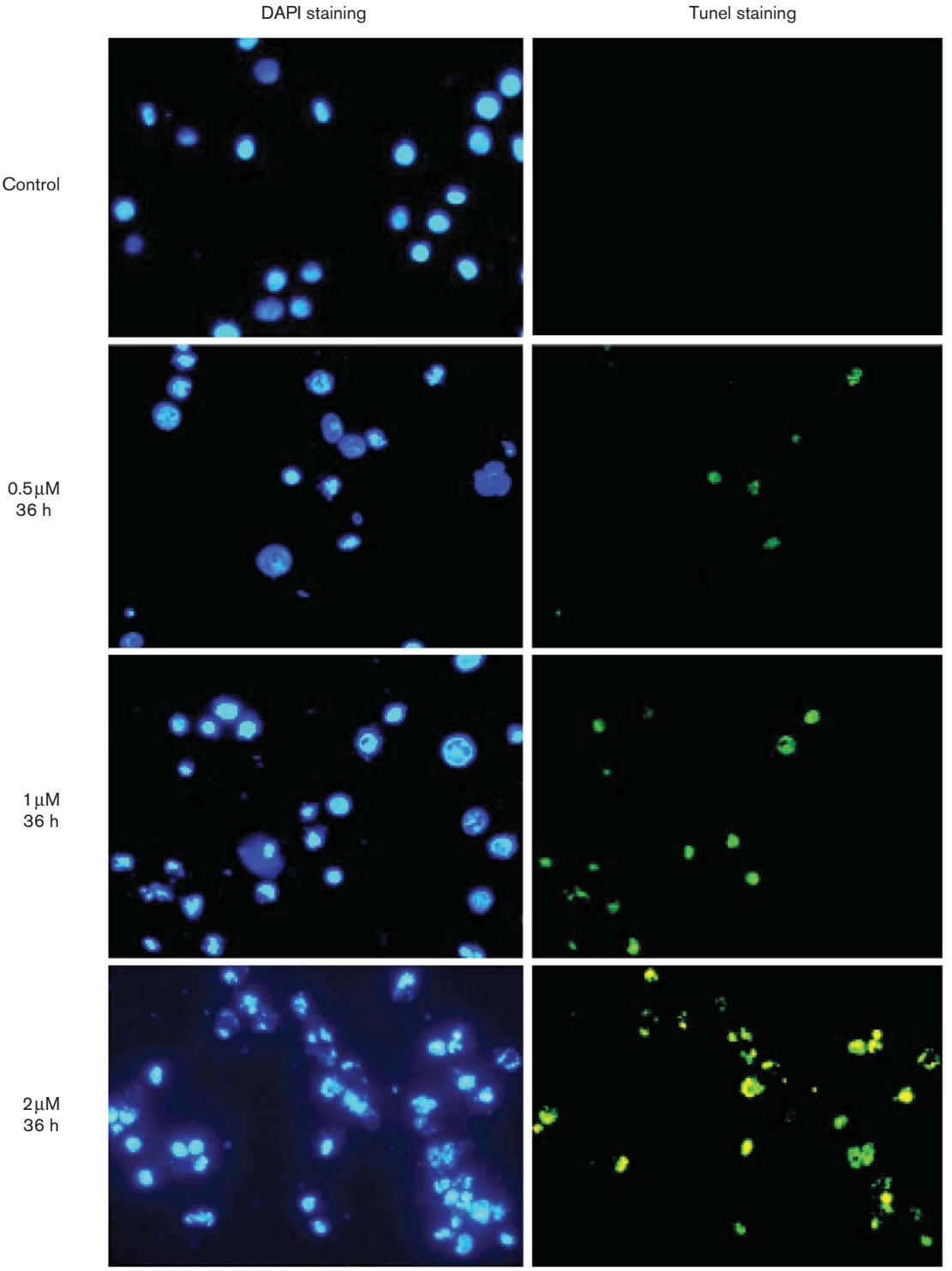
Deregulated expression of RTKs is a hallmark of malignancy. Inhibition of such RTKs has become a major focus of current anti-cancer drug development. Among them, the EGFR and VEGFR tyrosine kinase families belong to the best-studied and most attractive RTK targets for cancer chemotherapy [4–6]. Many data indicated that combined blockage on both EGFR/ErbB-2 and VEGFR (KDR and Flt-1) produced a more potent anti-tumor response [18].

We have now obtained 11,11′-dideoxy-verticillin, a naturally originating compound that combines EGFR/ErbB-2 as well as VEGFR-1 tyrosine kinase inhibition in the same molecule. In the cell-free tyrosine kinase assay, 11,11′-dideoxy-verticillin inhibited both EGFR and VEGFR-1 enzymes at low nanomolar $IC_{50}$ values. To our knowledge, 11,11′-dideoxy-verticillin is the most potent natural compound with tyrosine kinase inhibitory activity so far studied. Most of the natural tyrosine kinases inhibitors such as quercetin, genistein, erbstatin and herbimycin A displayed $IC_{50}$ values in the micromolar range. The potency of 11,11′-dideoxy-verticillin to inhibit RTK activity was confirmed at the cellular level, characterized by its significantly preventive effects on EGF-stimulated autophosphorylation of EGFR and HER2 in both MDA-MB-468 human breast carcinoma cell, and SK-OV-3 human ovarian adenocarcinoma cells as well (with a similar $IC_{50}$ around 2 μM). Of note, 11,11′-dideoxy-verticillin also inhibited VEGF-stimulated autophosphorylation of VEGFR-1 in human umbilical vein endothelial cells (HUVECs) with a similar submicromolar $IC_{50}$ value (data will be published in another paper).

Attempted investigations were further carried out to characterize the role of 11,11′-dideoxy-verticillin on major signal transduction pathways down-streaming EGFR and HER2, the Ras/mitogen-activated protein kinase (MAPK) pathway and the PI3K/AKT pathway, which are strictly associated with the proliferation and survival of cells. In fact, increased expression of activated Erk1/2 (p42/p44 MAPK) and AKT has been demonstrated in a number of human cancers [19,20]. It is noteworthy that 11,11′-dideoxy-verticillin potently inhibited EGF-induced phosphorylation of Erk1/2 in both MDA-MB-468 and SK-OV-3 cell lines. The inhibitory potency of 11,11′-dideoxy-verticillin for the Erk1/2 was similar to that for EGFR or HER2, respectively. This notion strongly implies that phosphorylation inhibition of 11,11′-dideoxy-verticillin on Erk1/2 might largely result from its antagonizing impact on phosphorylation of EGFR or HER2. 11,11′-Dideoxy-verticillin, however, exhibited no effect on EGF-induced phosphorylation of AKT in both MDA-MB-468 and SK-OV-3 cell lines. The discrepancy obtained in MDA-MB-468 cells is probably due to the fact that MDA-MB-468 cells hold a deletion of the PTEN tumor suppressor proteins, which consequently arrest the activation of AKT regardless of the presence of EGFR inhibitors [21].

Besides the critical roles of EGFR/ErbB-2 in the development and progression of tumors, the VEGFR family (KDR and Flt-1) is a key RTK highlighting the regulation of tumor angiogenesis [22]. Anti-angiogenic therapy through inhibition of VEGFR (KDR and Flt-1)-mediated effects is indeed expected to be one of the most promising therapeutic strategies. 11,11′-Dideoxy-verticillin potently inhibited the phosphorylation of VEGFR-1 in the biochemical and cell-based assay. Moreover, another study in our laboratory found that HUVECs were much more sensitive to 11,11′-Dideoxy-verticillin, compared to another normal lung fibroblast cell line WI-38, and combated several key steps involved in angiogenesis including migration and tube formation of HUVECs at low concentration without obvious cytotoxicity. Most importantly, 11,11′-dideoxy-verticillin significantly

**Fig. 5**




Induction of apoptosis by 11,11′-dideoxy-verticillin in MDA-MB-468 human breast carcinoma cells. MDA-MB-468 cells were incubated with different concentrations of 11,11′-dideoxy-verticillin for various time periods and harvested. Apoptosis of MDA-MB-468 cells in the same field were concurrently detected by DAPI staining (left panels, × 200) and the TUNEL method (right panels, × 200). Photographs are representative of three independent experiments.

**Table 3 Effect of 11,11′-dideoxy-verticillin on cell cycle distribution in MDA-MB-468 breast carcinoma cells**

| 11,11′-Dideoxy-verticillin (nM) | $G_0/G_1$ (%) | S (%) | $G_2/M$ (%) |
|---|---|---|---|
| 0 | 52.02 | 33.48 | 14.50 |
| 62.5 | 54.14 | 32.72 | 13.14 |
| 125 | 45.53 | 34.70 | 19.77 |
| 250 | 35.45 | 34.80 | 29.75 |
| 500 | 29.51 | 30.68 | 39.81 |

MDA-MB-468 cells were incubated in the absence or presence of 62.5–500 nM 11,11′-dideoxy-verticillin for 24 h. The cells were harvested and fixed with 70% ethanol for flow cytometry analyses. Data shown are representative of three independent experiments.

suppressed VEGF-induced tyrosine phosphorylation of both Flt-1 and KDR. This inhibition of receptor phosphorylation was correlated with a marked decrease in VEGF-triggered ERK activation and a dramatic increase in phospho-p38 MAPK (data to be published in another paper). The antagonizing function of 11,11′-dideoxy-verticillin on VEGFR-1, together with its other data on KDR in our laboratory, positively support that its anti-angiogenetic action was tyrosine kinase dependent. This subsequently promised its potential anti-tumor activity.

Since it is expected that combined fighting against both EGFR and VEGF pathways will have an element of anti-angiogenic activities and direct effects on tumor cell proliferation as well, we further investigated the anti-tumor effects of 11,11′-dideoxy-verticillin. Experimental results showed that it exhibited potent anti-tumor activity both *in vitro* and *in vivo*. The anti-tumor activities of 11,11′-dideoxy-verticillin were closely associated with its ability to counteract tumor cell proliferation, arrest cell cycle in the $G_2$/M phase and trigger apoptosis. Here, another finding should not be neglected: suppression of 11,11′-dideoxy-verticillin on growth of cancer cells by arresting the cell cycle and induction of cell apoptosis occurred at concentrations lower than that on phosphorylation of EGFR, HER2 and Erk1/2. These notions might, in turn, implicate the existence of the involvement of other anti-tumor mechanisms besides RTK-dependent pathways. 11,11′-Dideoxy-verticillin is a member of a large chemical class of compounds called epidithiodioxopiperazines (ETPs). In previous reports, some members of this family have been shown to be cytotoxic. Nanomolar concentrations of gliotoxin have been reported to be able to inhibit the activation of transcription factor NF-κB in response to a variety of stimuli in T and B cells [23]. Nanomolar verticillin derivative Sch52900 has been reported to be able inhibit the activation of AP-1 [24]. Therefore, in future research, the cytotoxic effects and detailed mechanism of 11,11′-dideoxy-verticillin merit further elucidation.

In addition, ETPs are characterized by a bridged disulfide diketopiperazine ring. The intact disulfide bridge has been shown to be essential for the toxicity of most ETP toxins in a previous study [25]. In our study, we found that in the tyrosine kinase assay, with the existence of 1 mM DTT, 11,11′-dideoxy-verticillin still showed strong inhibitory activity against EGFR and VEGFR-1, which implied that some structure other than the disulfide bridge might contribute to the tyrosine kinase inhibitory activity of 11,11′-dideoxy-verticillin.

In conclusion, this is the first report to disclose that 11,11′-dideoxy-verticillin, a compound of the novel ETP structural class from traditional Chinese medicine, exhibited potent dual inhibition on both EGFR/ErbB-2 and VEGFR-1 tyrosine kinase, and thus anti-tumor activity. Further study on the influence on other RTKs, structure–activity relationships and the enzyme kinetics of 11,11′-dideoxy-verticillin will prove the basis for effective anti-tumor therapies.

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