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Vaticaffinol, a resveratrol tetramer, exerts more preferable immunosuppressive activity than its precursor *in vitro* and *in vivo* through multiple aspects against activated T lymphocytes

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ABSTRACT

In the present study, we aimed to investigate the immunosuppressive activity of vaticaffinol, a resveratrol tetramer isolated from *Vatica mangachapoi*, on T lymphocytes both *in vitro* and *in vivo*, and further explored its potential molecular mechanism. Resveratrol had a wide spectrum of healthy beneficial effects with multiple targets. Interestingly, its tetramer, vaticaffinol, exerted more intensive immunosuppressive activity than resveratrol. Vaticaffinol significantly inhibited T cells proliferation activated by concanavalin A (Con A) or anti-CD3 plus anti-CD28 in a dose- and time-dependent manner. It also induced Con A-activated T cells undergoing apoptosis through mitochondrial pathway. Moreover, this compound prevented cells from entering S phase and G2/M phase during T cells activation. In addition, vaticaffinol inhibited ERK and AKT signaling pathways in Con A-activated T cells. Furthermore, vaticaffinol significantly ameliorated ear swelling in a mouse model of picryl chloride-induced ear contact dermatitis *in vivo*. In most of the aforementioned experiments, however, resveratrol had only slight effects on the inhibition of T lymphocytes compared with vaticaffinol. Taken together, our findings suggest that vaticaffinol exerts more preferable immunosuppressive activity than its precursor resveratrol both *in vitro* and *in vivo* by affecting multiple targets against activated T cells.

Introduction

Immunosuppressants are usually used for the treatment of a variety of immunologic disorders such as transplant rejection, rheumatic arthritis, systemic lupus erythematosus and multiple sclerosis. However, most of the immunosuppressive agents currently available, including glucocorticoids, cyclophosphamide and even cyclosporine A, have been reported to inevitably possess severe side effects primarily owing to poor selectivity (Hackstein and Thomson, 2004; Halloran, 2004; Kahan, 2003). Therefore, there is an urgent need for new potent immunosuppressive agents with negligible or acceptable toxicity.

Plant-derived natural products occupy a very important position in the area of drug discovery. Molecules such as paclitaxel, camptothecin, artemisinin, triptolide and curcumin are invaluable contributions of nature to modern medicine (Corson and Crews, 2007; Gautam and Jachak, 2009; Li and Vederas, 2009). However, the quest to find out novel effective natural remedies for the treatment of diseases is a never-ending venture. Recently, some substantially immunosuppressive and less-toxic polyphenols have received increasing attention. For example, dalesconols A and B were identified as novel immunosuppressive polyphenols with higher selectivity (Zhang et al., 2008). Astilbin isolated from the *Smilax glabra rhizome* was proved to be an immunosuppressive flavonoid unique in its selective inhibition on activated T lymphocytes (Fei et al., 2005). Those findings encouraged us to search for promising immunosuppressants from the Dipterocarpaceae family, which were shown to be abundant in bioactive polyphenols (Ge et al., 2006, 2008, 2009, 2010). Dipterocarpaceous plants are well known as rich sources of various resveratrol (3,5,4′-trihydroxystilbene) oligomers (Shen et al., 2009), many of which exhibit remarkable bioactivities such as antioxidant (Ge et al., 2009), antitumor (Ge et al., 2006), immunosuppressive (Ge et al., 2010) and acetylcholinesterase inhibitory (Ge et al., 2008) effects. Previously, it was reported that resveratrol, a well-known polyphenol, exhibited anticancer, antioxidant, anti-aging, chemopreventive, cardioprotective, neuroprotective, anti-inflammatory and immunomodulatory activities (Albani et al., 2010; Bereswill et al., 2010; Das and Das, 2010; Juan et al., 2012; Kang et al., 2009; Ungvari et al., 2010). In the present study, we found that vaticaffinol, a resveratrol tetramer isolated from an ethyl acetate extract of the branches and twigs of *V. mangachapoi* (Qin et al., 2011), exerted a more potent immunosuppressive activity than resveratrol both *in vitro* and *in vivo*. The mechanism of vaticaffinol's immunosuppressive effect on T cells involves cell cycle arrest, mitochondrial apoptosis and inhibition of ERK/AKT signalings.

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Materials and methods

Mice. Female BALB/c mice (6–8 weeks old, 18–22 g) were obtained from the Experimental Animal Center of Yangzhou University (Yangzhou, China). They were maintained with free access to pellet food and water in plastic cages 21 ± 2 °C and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

Cells and reagents. Mouse $CD3^+$ T cells from lymph nodes of BALB/c mice were purified using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) via magnetic cell separation with more than 98% purity. T cells were incubated in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. The following drugs and reagents were used: vaticaffinol ((2S, 2′R, 3R, 3′E, 4E, 8E)-1-O-β-D-glucopyranosyl-2-N-(2′-hydroxy-18-3′-alkenyl)-3-hydroxy-9-methyl-4,8-sphingadienine, 99.5% of purity, isolated and identified as reported previously (Qin et al., 2011)). Resveratrol, concanavalin A (Con A), ionomycin, 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), and carboxyfluorescein diacetate succinimidyl ester (CFSE) were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A (CsA) was purchased from Sandoz Ltd. (Basel, Switzerland). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-benzimida-zolcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). Picryl chloride (PCl) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Purified anti-mouse CD3 (145-2C11) and purified anti-mouse CD28 (37.51) were purchased from BD PharMingen (San Diego, CA). Annexin V-FITC/PI kit was purchased from BD Biosciences (San Jose, CA). Antibodies against PARP, caspase-3, Bcl-2, ERK, phospho-ERK (Thr 202/Tyr 204), AKT, phospho-AKT (Thr 308), PI3K, BAD, and p-BAD (Ser136) were purchased from Cell Signal Technology (Beverly, MA). Antibodies against Tubulin and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell proliferation assay. Lymph nodes cells were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium (0.2 ml) and treated with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 24, 48 and 72 h in the presence of Con A (5 μg/ml) or anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml). MTT (4 mg/ml in PBS, 20 μl per well) was added to each well 4 h before the end of incubation. Then culture media was removed and 200 μl DMSO was added to dissolve the crystals. The absorption values at 540 nm were measured. In some cases, cell proliferation was also determined by carboxyfluorescein diacetate succinimidyl ester (CFSE) assay as we previously reported (Sun et al., 2010). Briefly, purified T cells (1×10^7 cells per ml) from lymph nodes of BALB/c mice were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin and labeled with 2.5 μM CFSE for 10 min at 37 °C. After labeling, the cells were washed three times in 15% fetal bovine serum medium and resuspended in RPMI 1640 medium (1×10^6 cells per ml). The CFSE-labeled cells were cultured with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 48 h in the presence or absence of 5 μg/ml Con A.

Cell mitochondrial membrane potential assay. Cells were seeded in 12-well plates at a density of 1×10^6 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 48 h in the presence of Con A (5 μg/ml). Disruption of mitochondrial membrane potential was measured using JC-1 staining (10 μg/ml) as previously reported (Sun et al., 2009).

Subcellular fractionation. In some experiments, cells were separated into cytosolic and mitochondrial fractions using the ProteoExtract Cytosol/Mitochondria Fractionation Kit (Merck Bioscience, Bad Soden, Germany) according to the procedures provided by the manufacturer.

Cell apoptosis assay. Cells were seeded in 12-well plates at a density of 1×10^6 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 48 h in the presence of Con A (5 μg/ml). Cultured cells were stained with Annexin V-FITC and propidium iodide (PI) in the dark at 4 °C for 30 min and analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

Cell cycle assay. Cells were seeded in 12-well plates at a density of 1×10^6 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 48 h in the presence of Con A (5 μg/ml). Cell cycle was examined as we previously reported (Luo et al., 2011). Briefly, cultured cells were fixed in 75% ethanol at 4 °C overnight, and then stained with 50 μg/ml of PI containing 100 μg/ml of RNase A and 1% TritonX-100 in the dark at room temperature for 45 min. DNA contents of stained cells were analyzed with Modfit software (Becton Dickinson, San Jose, CA, USA).

Western blot. Cells were seeded in 6-well plates at a density of 1×10^7 cells/well in RPMI 1640 medium and treated with 1, 3, 10 μM vaticaffinol or 10 μM resveratrol for 24 h in the presence of Con A (5 μg/ml). Proteins lysed from cultured cells were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). After blocked in 5% nonfat milk at room temperature for 1 h, membranes were incubated with aimed primary antibodies at 4 °C overnight, and then with a horseradish-coupled secondary antibody at room temperature for 1.5 h. Final detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

Picryl chloride (PCl)-induced contact hypersensitivity. On the first day (day 0), female BALB/c mice were shaved and sensitized by painting 0.1 ml of 1% PCl in absolute ethanol on the naked skin of their abdomens. Five days later (day 5), vaticaffinol (10, 30 mg/kg) and resveratrol (10 mg/kg) dissolved in olive oil were given i.p., and then mice were challenged by painting 30 μl of 1% PCl in olive oil on right ear lobe. Eighteen hours later, ear thickness of right against left was measured with a digimatic micrometer (0.001 mm, Mitutoyo Co., Tokyo, Japan). The control mice were run parallel with other groups except for intraperitoneal (i.p.) administration of same volume of olive oil (Luo et al., 2011).

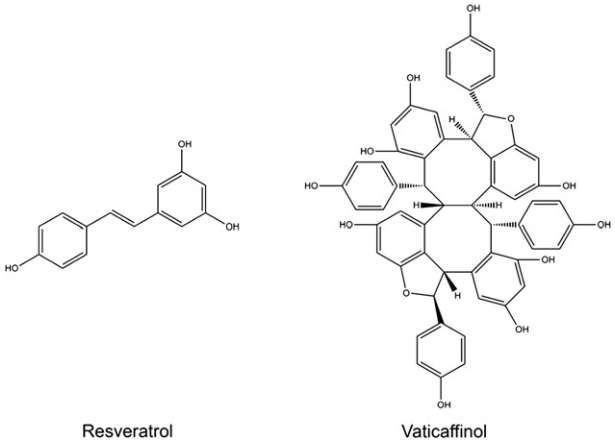

Fig. 1. Chemical structures of resveratrol and its tetramer vaticaffinol.