Poria cocos polysaccharide attenuates RANKL-induced osteoclastogenesis by suppressing NFATc1 activity and phosphorylation of ERK and STAT3

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Running title: PCP suppresses RANKL-induced osteoclastogenesis

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Abstract: Pathological fractures caused by osteolytic lesions seriously threaten the health of patients. Osteoclasts play important roles in bone resorption whose hyperfunction are closely related to osteolytic lesions. Studies on osteoclast differentiation and function assist in the prevention of excessive bone loss associated diseases. We screened a variety of natural compounds with anti-inflammatory effect and found that poria cocos polysaccharide (PCP) inhibited RANKL-induced osteoclast formation and bone resorption via TRAcP staining, immunofluorescence, RT-PCR and western blot. PCP down-regulated phosphorylation of STAT3, ERK and JNK, and thus repressed the expression of NFATc1 and c-Fos during RANKL-induced osteoclastogenesis. Besides, the expression of bone resorption related genes such as TRAcP and CTSK was suppressed by PCP. The results suggest that PCP can be invoked as a candidate for the treatment of osteolytic diseases by inhibiting osteoclastogenesis.

Keywords: Poria cocos polysaccharide; Osteoclast; Bone resorption; ERK; STAT3

38 **Introduction**

39 The bone is a dynamic, and constantly remodeling tissue. Bone remodeling results from the action of
40 osteoclasts and osteoblasts, in which osteoclasts resorb mineralized bone followed by the formation
41 of bone matrix through the osteoblasts that subsequently become mineralized [1]. In a homeostatic
42 equilibrium resorption and formation are balanced so that old bone is continuously replaced by new
43 tissue. Disturbance of the balance between bone resorption and bone formation can result in
44 pathological abnormalities in bone structure, such as osteoporosis and osteolytic lesions [2].
45 Osteolytic lesions, could be caused by various disorders of the bones such as simple bone cyst,
46 aneurysmal bone cyst, plasmacytoma, giant cell tumor, eosinophilic granuloma and tuberculosis, are
47 mainly characterized by sthenic bone resorption and often associated with severe pain, pathological
48 fracture [3]. Osteoclasts are the major effector cells responsible for bone resorption, the rate of bone
49 resorption can be regulated either by changing the number or the activity of mature osteoclasts [4, 5].
50 Therefore, in order to explore new treatments for osteolytic lesions, it is critical to investigate the
51 therapeutic effects of natural compounds on the differentiation and function of osteoclasts.

52

53 Derived from hematopoietic stem cells, osteoclasts are gradually differentiated and fused to form
54 multinucleated cells under the action of macrophage colony stimulating factor (M-CSF), receptor
55 activator of nuclear kappa B ligand (RANKL) and so on [6, 7]. RANKL binds to the receptor RANK
56 on the osteoclast membrane and recruits adaptor molecules such as TRAF6 to regulate the
57 downstream signaling pathway [8, 9]. Then the nuclear transcription factors AP-1 and NFATc1 are
58 activated to promote cell differentiation and expression of bone resorption related genes such as

tartrate resistant acid phosphatase (TRAcP), matrix metalloproteinase 9 (MMP 9), integrin beta 3 and cathepsin K (CTSK) [10-12]. Inhibitory of RANKL induced osteoclastogenesis and bone resorption activity is the key issue for the treatment of osteolytic disease.

Poria is a fungus in the family *Polyporaceae* with a subterranean growth habit. The chemical compositions of *Poria cocos* mainly include triterpenes, steroids, amino acids, polysaccharides, and so on [13]. In recent studies, *Poria cocos* polysaccharide (PCP) has been shown to exhibit many beneficial biological activities including anti-inflammatory and anti-oxidation effects [14, 15]. Treatment with PCP significantly induces NO production and iNOS transcription by triggering the activation of NF- κ B/Rel through p38 kinase pathway in the mouse macrophage line RAW 264.7 [16, 17]. However, the effect of PCP on osteoclast differentiation and function is unclear. Therefore, we studied the regulation of PCP on RANKL induced osteoclastogenesis of bone marrow derived macrophages (BMMs) and its underlying mechanism. We found that PCP suppressed osteoclast differentiation and bone resorption activity by down-regulating phosphorylation of STAT3, ERK and JNK. The results suggest that PCP could be used as a promising novel candidate for the treatment of osteolytic disease.

76 **Materials and methods**

77 *Materials*

78 Alpha modified minimal essential medium (α -MEM) and fetal bovine serum (FBS) were purchased
79 from Thermo Fisher Scientific (Scoresby, Australia). PCP (purity >98%) was purchased from Solarbio
80 (Beijing, China) and prepared at a stock concentration of 1mM in phosphate buffered saline (PBS).
81 Antibodies specific for Integrin- β 3, c-Fos, CTSK, NFATc1, STAT3, ERK, JNK, phosphorylated (p)
82 ERK, p-STAT3, p-JNK and β -actin were obtained from Santa Cruz Biotechnology (San Jose, CA).
83 Antibodies to V-ATPase d2 was produced as previously described [18]. The MTS and luciferase
84 assay system were obtained from Promega (Sydney, Australia). Recombinant macrophage colony
85 stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN). Leucocyte acid
86 phosphatase staining kits were obtained from Sigma-Aldrich (Sydney, Australia). Recombinant
87 GST-rRANKL protein was expressed and purified as previously described [19].

88

89 *Cell culture*

90 RAW264.7 cells (mouse macrophage cells) were obtained from the American Type Culture
91 Collection (Manassas, VA) and cultured in α -MEM supplemented with 10% FBS, 2 mM L-glutamine,
92 100 U/mL penicillin, and 100 μ g/mL streptomycin (complete medium). Bone marrow monocytes
93 (BMMs) were obtained from 6-week-old C57BL/6J mice, which were euthanized according to
94 procedures approved by the Animal Ethics Committee of the University of Western Australia
95 (RA/3/100/1244). Long bones were dissected free of soft tissues and the bone marrow was flushed

from the femur and tibia, and then cultured in complete medium in the presence of M-CSF (50 ng/mL).

Drug screening assay and osteoclastogenesis assay

Drug screening assays were conducted using BMMs isolated as described above to evaluate RANKL-induced osteoclastogenesis. BMMs were plated into 96-well culture plates at a density of 6×10^3 cells/well, and treated with complete medium containing M-CSF and GST-rRANKL (50 ng/mL) in the presence or absence of natural compounds (screening assay 10 μ M) or varying concentrations of PCP. The cell culture medium was changed every 2 days. After 5 days, cells were fixed with 4% paraformaldehyde for 10 min, washed three times with PBS, and then stained for tartrate resistant acid phosphatase (TRAcP) enzymatic activity using leucocyte acid phosphatase staining kit, following the manufacturer's procedures. TRAcP-positive multinucleate cells (> 3 nuclei) were counted as osteoclasts.

Cytotoxicity assays

BMMs were seeded into a 96-well plate at 6×10^3 cells/well and incubated overnight to adhere. Next day, the cells were co-cultured with varying concentrations of PCP for 48 h. Then 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution (20 μ L/well) was added into each well, and incubated with cells for additional 2 h. The absorbance at 490 nm of each well was detected using a microplate reader (Multiscan Spectrum, Thermo labsystem, Chantilly, VA).

117

118 *Immunofluorescent staining*

119 BMMs were seeded at a density of 6×10^3 cell/well in the presence of M-CSF (50 ng/mL) and
120 incubated for 12 h to adhere. Cells were then stimulated with M-CSF and GST-rRANKL (50 ng/mL)
121 until mature osteoclasts formed. The osteoclasts were treated with different concentrations of PCP
122 for 48 h before being fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 PBS,
123 and then blocked with 3% BSA in PBS. Next, cells were incubated with Rhodamine-conjugated
124 phalloidin for 45 minutes in the dark to stain F-actin. Cells were then washed with PBS, nuclei were
125 counter stained with DAPI, and mounted for confocal microscopy.

126

127 *Hydroxyapatite resorption assay*

128 To measure osteoclast activity, osteoclasts were first generated from BMMs (1×10^5 cells per well)
129 cultured onto 6-well collagen coated plates (BD Biocoat, Thermo Fisher, Scoresby, Australia) and
130 stimulated with 50 ng/mL GST-rRANKL and M-CSF until mature osteoclasts were generated [20].
131 Cells were gently detached from the plate using cell dissociation solution (Sigma-Aldrich, Sydney,
132 Australia) and mature osteoclasts were seeded into individual wells in hydroxyapatite coated
133 96-well plates (Corning Osteoassay, Corning, NY) at equal numbers. Mature osteoclasts were
134 incubated in medium containing GST-rRANKL and M-CSF with or without PCP at the indicated
135 concentration. Forty-eight hours later, half of the wells were histochemically stained for TRAcP
136 activity as above to assess the number of multinucleated cells per well. The remaining wells were
137 bleached for 10 min to remove the cells and allow measurement of the resorbed areas. Resorbed

areas were photographed under standard light microscopy and the percentage area of hydroxyapatite surface resorbed by the osteoclasts was quantified though Image J software (NIH, Bethesda, MD).

Luciferase reporter assays

To investigate NFATc1 transcriptional activation, RAW 264.7 cells were stably transfected with an NFATc1 responsive luciferase reporter construct [21, 22]. Transfected cells were cultured in 48-well plates at a density of 5×10^4 cells/well and pretreated with various concentrations of PCP for 1 h. Following pre-treatment cells were subsequently stimulated with GST-rRANKL (50 ng/mL) for 24 h and luciferase activity was detected using the luciferase reporter assay system according to the manufacturer's protocol (Promega, Sydney, Australia).

Quantitative RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Scoresby Australia). The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with 1 μ g of RNA template and oligo-dT primers. Polymerase chain reaction amplification of specific sequences was performed using the following cycle: 94°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 60°C for 40 sec, and 72°C for 40 sec, and a final extension step of 5 min at 72°C. The detailed information of specific primers is shown in **Table 1**. The relative mRNA level was calculated by normalization to *Actb* and *B2m*.

Western blotting

BMMs were cultured in complete medium with M-CSF in 6-well plates and stimulated with 50 ng/mL GST-rRANKL for the stated times. Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer, and proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Silverwater, Australia). The membranes were blocked in 5% skim milk for 1 h, and then probed with various specific primary antibodies with gentle shaking overnight at 4 °C. Membranes were washed and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibody reactivity was then detected with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ), visualized on an Image-quant LAS 4000 (GE Healthcare, Silverwater, Australia).

Statistics

All data are representative of at least three experiments of similar results performed in triplicate unless otherwise indicated. Data are expressed as mean $\pm$ SD. One-way ANOVA followed by Student-Newman-Keuls post hoc tests was used to determine the significance of difference between results, with p value < 0.05 being regarded as significant.

Results

PCP impairs RANKL-induced osteoclast formation

Osteoclastogenesis assay was performed to screen dozens of natural compounds at a concentration of 10 μ M for their ability to suppress RANKL-induced osteoclast formation from mouse BMM [23, 24]. Among examples of candidate natural compounds (**Table 2**), PCP was identified as exhibiting an inhibitory effect on RANKL-induced osteoclastogenesis. To determine if the effect of PCP on osteoclast formation is dose-dependent, BMMs were treated with RANKL and M-CSF for 5 days with different concentrations of PCP as indicated in Figure 1. Through TRAcP staining, we found that PCP presented an inhibitory effect on RANKL-induced osteoclastogenesis. The results showed that PCP dose-dependently decreased the number of TRAcP positive cells in each well (96-well plate) with significant inhibition occurring at a concentration of 5 μ M and higher (**Figure 1A, B**). Then, toxicity of PCP was evaluated by an MTS assay. The results demonstrated that PCP did not cause cell loss at a concentration of 0.5 μ M or above (**Figure 1C**). Therefore, the suppression of PCP on RANKL induced osteoclastogenesis was not a result of toxicity.

Next, in order to investigate the effects of PCP on osteoclast fusion, rhodamine-phalloidin and DAPI staining were used to determine the number of nuclei per osteoclast that formed in the presence or absence of varying doses of PCP (**Figure 2A**). The results showed that PCP treatment at the concentrations of 5 and 8 μ M significantly reduced osteoclast number as well as the average number of nuclei per osteoclast (**Figure 2B, 2C**) which was consistent with TRAcP staining. Taken together, PCP has a dose-dependent inhibitor effect on RANKL-induced osteoclastogenesis and cell fusion.

PCP suppresses RANKL-induced osteoclastic hydroxyapatite resorption activity

Hydroxyapatite resorption assay was performed to evaluate the effect of PCP on osteoclastic resorption activity (**Figure 3A**). The results showed that groups treated with PCP at concentrations of 5 μ M and 8 μ M presented distinct decrease in hydroxyapatite resorption area compared to control groups, while there were no significant differences in the number of osteoclasts between these two groups (**Figure 3B, 3C**). Thus, these data revealed that resorption activity of osteoclasts was attenuated by PCP.

PCP attenuates osteoclast and bone resorption related genes expression

Our data demonstrated that PCP has an inhibitory effect on RANKL-induced osteoclastogenesis and osteoclastic resorption activity. To further explore their mechanism, RT-PCR was performed to screen the altered osteoclast related genes. The results showed that genes expression of *NFATc1* and *c-Fos* were decreased when the concentration of PCP reached 8 μ M and 5 μ M, respectively (**Figure 4A, 4B**). Furthermore, we detected the regulation of PCP on bone resorption related genes, and found that genes expression of *Ctsk* and *Acp5* were remarkably down-regulated by the treatment of PCP (**Figure 4C, 4D**).

PCP restrains the activity of NFATc1 and downstream protein expression

The effect of PCP on RANKL-induced NFATc1 activity was detected through luciferase reporter assay using RAW 264.7 cell line stably transfected with NFATc1 reporter gene. We found that RANKL-induced NFATc1 activity was repressed by PCP at the concentration of 5 and 8 μ M (**Figure**

5A). In addition, the results of western blotting showed that RANKL-induced up-regulation of NFATc1 expression in BMMs on day 3 and 5 was significantly restricted by PCP (Figure 5B). Furthermore, the expression of c-Fos, one of the regulators of NFATc1, and bone resorption associated proteins such as CTSK and Integrin β 3 was also down regulated in the presence of PCP (Figure 5B).

PCP inhibits phosphorylation of ERK, JNK and STAT3 during osteoclastogenesis

To further explore the potential mechanisms of PCP in osteoclastogenesis, we investigated the effect of PCP on RANKL-mediated JNK and ERK signaling pathways by western blot. The results showed that the activation of JNK1/2 was inhibited in the presence of PCP. Besides, PCP at a concentration of 8 μ M suppressed ERK1/2 phosphorylation significantly at 60 min. Furthermore, we determined the effect of PCP on RANKL-stimulated STAT3 signaling level. The results showed an inhibitory effect of PCP on the phosphorylation of STAT3 at 30 and 60 minutes compared to control groups (Figure 6). Therefore, these data indicated that PCP inhibited RANKL-induced osteoclast formation and function by regulating the activities of JNK, ERK and STAT3.

234 Discussion

235 Polysaccharide and their derivatives extracted from *Poria cocos*, has the ability to modulate cellular
236 immunity [25]. It has been reported that traditional Chinese herbal medicines consisting of *Poria*
237 *cocos* could alleviate osteoporosis induced by ovariectomy through Wnt/ β -catenin signaling
238 pathway of osteoblast [26] and prevent bone loss [27]. In this study, we found that PCP
239 down-regulates RANKL-induced osteoclastogenesis through suppressing both the number and size
240 of osteoclasts. Meanwhile, resorption activity of mature osteoclasts was significantly restrained by
241 PCP, indicating that PCP not only affected the genesis of osteoclasts, but also affected its function.
242 Investigation of mechanism revealed that PCP represses NFATc1 pathway by reducing the
243 phosphorylation of ERK, JNK and STAT3 in osteoclast. These results indicate that PCP may be a
244 potential drug used for preventing osteolytic lesions.

245

246 In terms of bone absorption, osteoclasts are in contact with the bone surface, followed by integrin
247 on the cell membrane recruited downstream proteins such as tyrosine kinases c-Src, Pyk2 and Syk
248 to induce osteoclast polarization [28]. Then the sealing zone is formed so that the osteoclasts are
249 tightly bound to bone surface [29]. Hydrogen ions are excreted through the proton pump and
250 contribute to form an appropriate environment with low pH in the resorption pits to promote
251 mineral degradation [30]. In addition, the degradation of the bone matrix also depends on the
252 various enzymes include CTSK and TRAcP [31]. CTSK degrades collagen I in an acidic
253 environment and TRAcP synergize CTSK to degrade organic matrix [32, 33]. This study found that
254 PCP significantly inhibited RANKL-induced osteoclastic bone resorption by suppressing Integrin

β3 and CTSK expression.

The expression of TRAcP, Integrin β3 and CTSK are regulated by AP-1 and NFATc1, two crucial transcription factors during osteoclastogenesis [34]. RANKL activates downstream molecules to dephosphorylate the cytoplasmic component of the NFATc1 to facilitate NFATc1 translocate into the nucleus to regulate osteoclastic specific gene expression [35]. As two key components of AP-1, c-Fos and c-Jun are induced at an early stage during osteoclast differentiation. Due to the defection of osteoclast lineage commitment, c-Fos-knockout mice develop severe osteopetrosis [36]. A study has found that HIF-2α inhibitor suppressed inflammatory responses and osteoclastogenesis by blocking the activation of c-Jun, and c-Fos [37]. Besides, exogenous IFN-β may reduce joint inflammation and bone destruction by inhibiting c-Fos expression during osteoclast formation [38]. Our research revealed that PCP repressed RANKL-induced expression of c-Fos and NFATc1 in osteoclastogenesis and then inhibited bone resorption related genes such as TRAcP and CTSK.

ERK and JNK belong to the subfamilies of mitogen activated protein kinase (MAPK), which is part of the important signaling pathways in eukaryotic cells and plays key roles in regulating cell structure and function [39]. AP-1 is regulated by ERK and JNK during osteoclast formation. Phosphorylated ERK enters the nucleus to regulate the transcription of c-Fos, whereas JNK increases the transcription activity of AP-1 by phosphorylating c-Jun [40]. It has been demonstrated that IL-6 regulated RANKL-induced osteoclast differentiation and activity through promoting phosphorylation of ERK and JNK [41]. As an indispensable key molecule for chronic inflammation

that promotes tumorigenesis, STAT3 regulates the biological behavior of cancer cells and immune cells [42]. Furthermore, STAT3 signaling is also involved in regulating osteoclastogenesis. A recent study showed that peroxiredoxin II negatively regulates lipopolysaccharide induced osteoclast formation via repressing STAT3 signaling [43]. We found that PCP reduced RANKL mediated phosphorylation of ERK, JNK and STAT3, thereby inhibited the expression of c-Fos and NFATc1 during osteoclast formation.

It has become an urgent public health issue for severe osteolytic diseases, such as osteoporosis. The targeted inhibitors against osteoclast formation and function have been used in clinics such as bisphosphonates [44]. However, potential side effects including atypical femur fractures and bisphosphonate-related osteonecrosis of the jaw are the constraints of above drugs [45-47], and traditional herbal medicines might serve as an alternative treatment option for skeletal diseases [48, 49]. This study might provide a new direction and choice for the treatment of osteolytic diseases.

290 **Competing Interests**

291 The authors declare no conflict of interest.

292

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Figure legends

Figure 1. PCP impairs osteoclast formation. **(A)** Representative images of BMMs stained for TRAcP treated with PCP at different dosages. Scale bar represents 200 μm . **(B)** Quantification of osteoclasts in each well (96-well plate). **(C)** Survival of BMMs in the presence of PCP as assessed by MTS cell viability assay. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 2. PCP inhibits RANKL-induced cell fusion. **(A)** Representative confocal images of osteoclasts stained for F-actin and nuclei. Scale bar represents 200 μm . **(B)** Quantification of osteoclasts in each field and average osteoclasts nuclei number in each field. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 3. PCP blocks osteoclast hydroxyapatite resorption activity. **(A)** Representative images of osteoassay surface 96-well plate after removal of osteoclasts (down) with corresponding TRAcP stained osteoclasts (up). Scale bar represents 200 μm . **(B)** Quantification of osteoclasts in each well (96-well plate). **(C)** Quantification of hydroxyapatite resorption area on the osteo surface. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 4. PCP attenuates the expression of osteoclastic marker genes. BMMs were treated with M-CSF (50 ng/mL) and GST-rRANKL (50 ng/mL) in the presence or absence of indicated concentrations of PCP. Gene expression was normalized to B2M. Relative mRNA expression levels of NFATc1 (A), c-Fos (B), CTSK (C) and TRAcP (D) during osteoclastogenesis. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 5. PCP decreases osteoclast and bone resorption - related gene expression on protein level. **(A)** Luciferase activity in RANKL stimulated RAW264.7 cells transfected with an NFATc1 luciferase construct. Transfected cells were pre-treated with indicated concentrations of PCP, and subsequently stimulated with GST-rRANKL (50 ng/mL) for 24 h. **(B)** Representative western blot images of c-Fos, NFATc1, V-ATPase-d2, CTSK, Integrin β 3 and ACTB from BMMs which were induced with RANKL (50 ng/mL) and M-CSF (50 ng/mL) in the presence of PCP. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as *** ($p < 0.001$).

Figure 6. PCP suppresses phosphorylation of JNK, ERK and STAT3. BMMs were induced with RANKL (50 ng/mL) and M-CSF (50 ng/mL) in the presence of PCP for 60 minutes. Western blot was probed with p-JNK1/2, JNK, p-ERK1/2, ERK, p-STAT3 and STAT3 specific antibodies and the phosphorylated proteins was normalized to unphosphorylated proteins.

Table 1: Primer sequences for qPCR

Genes	Forward (5'-3')	Reverse (5'-3')	Tm (°C)
<i>NFATc1</i>	GGAGAGTCCGAGAATCGAGAT	TTGCAGCTAGGAAGTACGTCT	61
<i>B2m</i>	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCTGGCTTCCCATTCT	61
<i>c-Fos</i>	GCGAGCAACTGAGAAGAC	TTGAAACCCGAGAACATC	60
<i>Ctsk</i>	GGGAGAAAAACCTGAAGC	ATTCTGGGGACTCAGAGC	61
<i>Acp5</i>	TGTGGCCATCTTTATGCT	GTCATTTCTTTGGGGCTT	61

NFATc1, nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1; *B2m*, beta-2 microglobulin; *c-Fos*, Fos proto-oncogene, AP-1 transcription factor subunit; *Ctsk*, cathepsin K; *Acp5*, tartrate-resistant acid phosphatase.

Table 2: The inhibitory effect of natural compounds on RANKL-induced osteoclastogenesis

Compounds name	Origins	Inhibitory effect on osteoclastogenesis (IC50)
Poria cocos polysaccharide (PCP)	Natural	IC50 $\approx$ 5 μ M
Dipsacoside B	Natural	IC50 > 10 μ M or no effect
Epigallocatechin gallate	Natural	IC50 > 10 μ M or no effect
Sinensetin	Natural	IC50 > 10 μ M or no effect
Scutellarein	Natural	IC50 > 10 μ M or no effect
Diosmetin	Natural	IC50 > 10 μ M or no effect
Ellagic Acid	Natural	IC50 > 10 μ M or no effect
Cryptochlorogenic Acid	Natural	IC50 > 10 μ M or no effect
Tetrodotoxin	Natural	Toxic
Diosmetin	Natural	IC50 > 10 μ M or no effect

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6