

In vitro comparison of anticancer and immunomodulatory activities of resveratrol and its oligomers

Jeffery F. Zhang¹, Everett Henry², L. Harris Zhang²

¹Morris County Vocational School, The Academy for Biotechnology, Mountain Lakes, NJ

¹Mountain Lakes High School, Mountain Lakes, NJ

²NanoBiotec, LLC, Hanover, NJ

SUMMARY

Stilbenoids are a group of naturally occurring phenolic compounds produced *de novo* in many plant species to protect against fungal infection and toxins. Resveratrol (3,4',5-trihydroxystilbene), a stilbenoid isolated from grapes and many species of plants, is widely known for its extensive bioactivities, such as antioxidation, anti-inflammatory and anticancer effects. Recently, many novel resveratrol oligomers have been isolated from various plants. While their structures are diverse, these oligomers are characterized by the polymerization of two or more resveratrol units. Little is known regarding the bioactivities of these novel resveratrol oligomers. We hypothesized that these oligomers might have similar or superior bioactivities to resveratrol. In this study, we designed *in vitro* models to compare resveratrol side by side with natural dimers (ϵ -viniferin and gnetin H) and trimers (suffruticosol B and C) from plants for their bioactivities. Our data showed that these oligomers, similar to resveratrol, had many activities such as downregulation of cancer cell proliferation, upregulation of normal keratinocyte growth, and the modulation of the expression of pro- and anti-inflammatory cytokines in immune cells. Furthermore, these oligomers appeared to have higher cytotoxicity than resveratrol in normal human blood cells; the order was the trimers (suffruticosol B and C) > dimers (ϵ -viniferin and gnetin H) > monomer (resveratrol). However, only suffruticosol C, gnetin H and resveratrol showed anticancer activity against human T98G glioblastoma cells at lower concentrations. No correlation between anticancer or immunomodulating effects and the degree of polymerization was observed. Our results provide some preliminary evidences that resveratrol oligomers could be potential preventive or therapeutic agents for cancers and other immune-related diseases. As far as any superiority of efficacy of these stilbenoids is concerned, more extensive studies are definitely needed.

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a well-known stilbenoid, a group of naturally occurring phenolic compounds found in many plant species. Stilbenoid are produced *de novo* in plants as antimicrobial compounds to protect against injury or attack by pathogens, such as

viruses, bacteria, or fungi (1). Rich sources of resveratrol in food include the skin of grapes, various berries, and others. It is commonly used as a dietary supplement and extensively studied in laboratory models of various human diseases, such as hypertension, diabetes, obesity, cancers, and others (2, 3). Resveratrol has been reported to exhibit a broad variety of biological and pharmacological activities ranging from antibacterial, antiviral, antioxidant, anti-inflammatory, immunomodulation, and cancer prevention (Figure 1) (3, 4, 5). For example, resveratrol can be used as complementary therapeutic for autoimmune diseases including multiple sclerosis and immune thrombocytopenic purpura due to its potential to reverse the imbalance of Th17/Treg cells (6, 7). Resveratrol was also reported to have chemopreventive activity on major stages of carcinogenesis (8). Currently multiple clinical trials of resveratrol are ongoing and have focused on cancer, neurological disorders and others. Therefore, it is clear that many beneficial effects of resveratrol have been observed. However, resveratrol's therapeutic efficacy depends on several factors such as types of cancer, demographics, etc. (1).

In the latest decade, many novel stilbenoids similar to resveratrol have been discovered in various plant species (3). These stilbenoids with complex unique structures exist as oligomers of resveratrol (Figure 2), resulting from polymerization of two or more resveratrol units (3, 5). There is little information, however, regarding the bioactivities of these novel resveratrol oligomers. We hypothesized that these oligomers, like resveratrol, might have anticancer and immunomodulating activities and potential utility as preventive

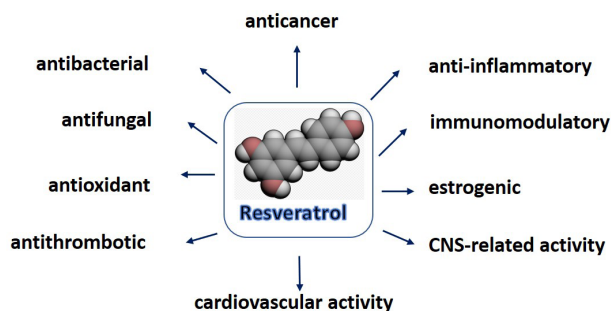


Figure 1. Pleiotropic biological and pharmacological activities of resveratrol. Resveratrol found in many plant and food is commonly used as a dietary supplement. It exhibits a broad variety of biological and pharmacological activities with potential for our wellness.

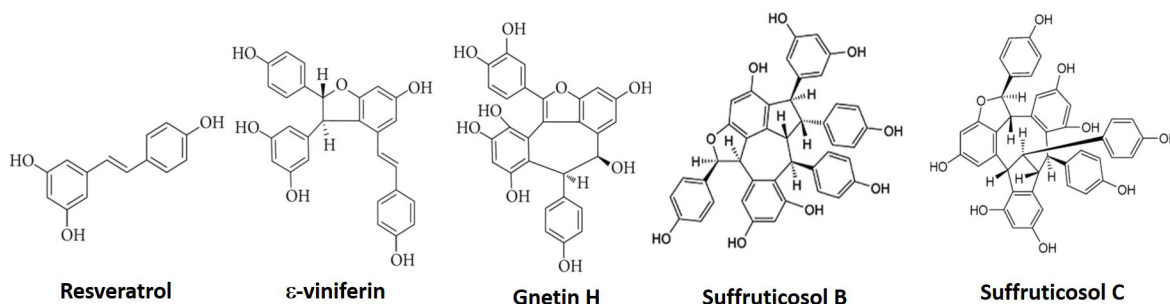


Figure 2. Chemical structures of resveratrol and its oligomers tested in this study. More and more novel stilbenoids similar to resveratrol have been discovered from plants. These stilbenoids have complex unique polyphenolic structures as oligomers of resveratrol.

or therapeutic agents for cancer and other immune-related diseases. It is of particular interest to examine whether these oligomers are comparable or superior to the monomer resveratrol in term of anticancer and immunomodulating activities.

RESULTS

To test our hypothesis, in the present study we designed several *in vitro* cell models to compare side by side the resveratrol versus two dimers and two trimers for their activities on several common cancer cell lines representing human solid and hematopoietic tumor cells, normal human skin cells (keratinocytes), as well as primary immune cells activated with two important stimuli mainly targeting T lymphocytes, monocytes, and B lymphocytes. Before investigation of these five stilbenoid compounds, we set out to test their potential cytotoxic concentrations on normal cells, using peripheral blood mononuclear cells (PBMCs) isolated from normal human blood. We estimated the cell viability by trypan blue staining and microscopic observation (data not shown) and also quantitated adenosine triphosphate (ATP) present in the cell culture, an indicator of metabolically active cells. We tested three concentrations (0.125, 1.25, 12.5 μ M) of the compounds. **Figure 3** shows that all these compounds

at lower concentrations (0.125-1.25 μ M) had no appreciable toxicity ($p>0.05$). While the dimer gnetin H and the trimers suffruticosol B and C at higher concentrations, $\geq 12.5 \mu$ M, resveratrol and e-viniferin at 12.5 μ M had no marked toxicity ($p>0.05$). The order of toxicity at 12.5 μ M is: suffruticosol C > suffruticosol B > gnetin H.

In next experiments, we chose the same non-toxic compound concentrations for normal PBMCs to test any specific cancer-killing activity of these stilbenoids. Five common lines of human cancer cells representing glioblastoma and lymphoma were tested: glioblastoma including A-172, T98G and U-87 cells; and lymphoma including Karpas-422 and RIVA cells. Among these tested cancer cell lines, we found that T98G was the most sensitive to these stilbenoids; proliferation of these cells was pronouncedly suppressed with the treatment of three stilbenoids, namely, resveratrol, the dimer e-viniferin and the trimer suffruticosol C at 1.25 μ M ($> 50\%$ inhibition, $p<0.01$), while gnetin H and suffruticosol B up to 1.25 μ M had no significant inhibition on T98G cells ($p>0.05$). Proliferation of the other lines of cancer cells was not clearly impacted by any of these compounds at 1.25 μ M (**Figure 4**), although there was significant inhibition of these cancer cells with compounds at 12.5 μ M or higher (data not shown).

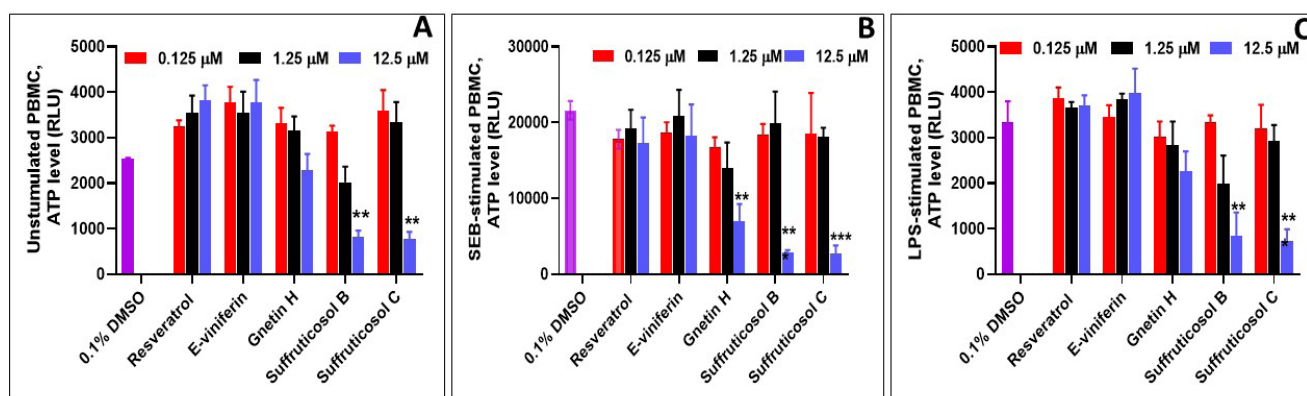


Figure 3. Effects of compounds on growth of human PBMC in the presence and absence of stimuli. Normal PBMCs were isolated from various donors of human blood and cultured in cell plates for 16 h and treated with serially diluted test compounds at concentrations ranging from 0.125-12.5 μ M. Following an additional 3 days (Panel A, unstimulated or Panel B, SEB stimulated PBMC) or 1 day (Panel C, LPS stimulated PBMC) of incubation, cell proliferation was determined. Results shown are means \pm SD from three experiments ($n=3$). Analyses for multiple group comparisons were performed with 1-way ANOVA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (compound treated vs 0.1% DMSO control).

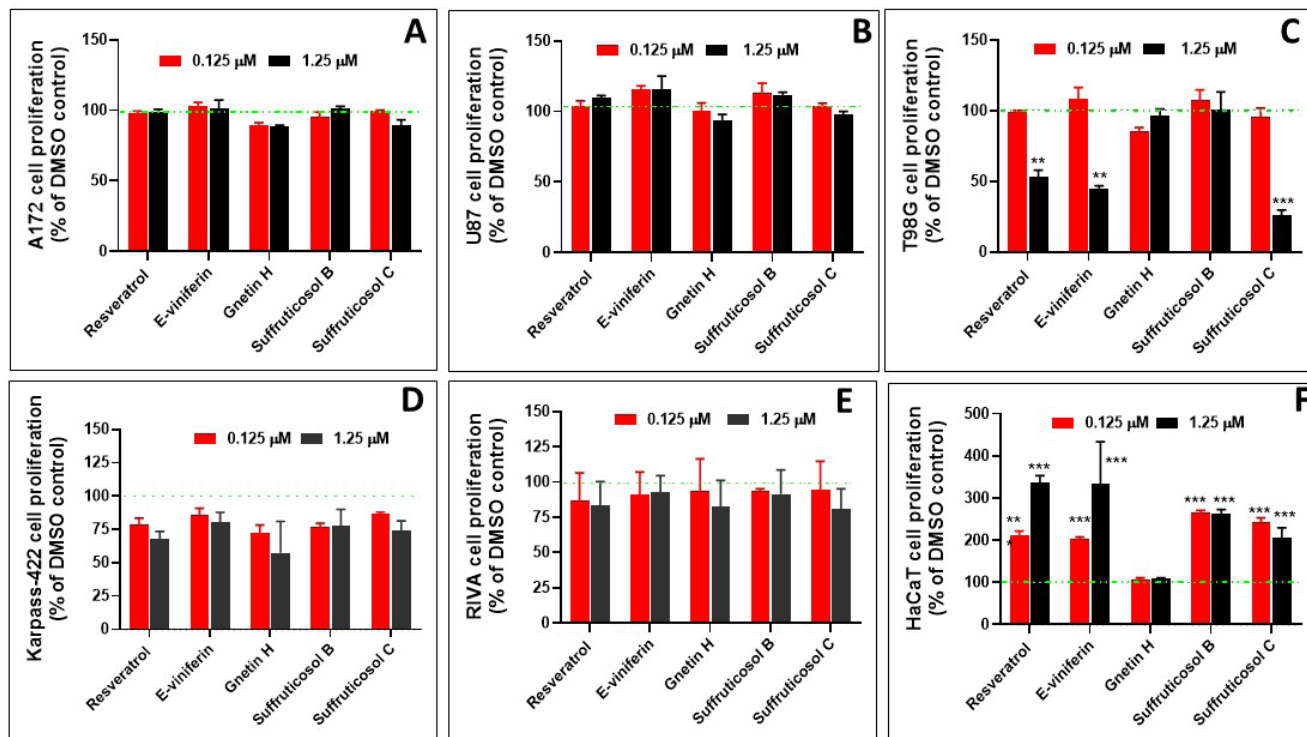


Figure 4. Effects of compounds on proliferation of various human cancer cells and keratinocytes from normal skin. Human cancer cells or skin cells were cultured in cell plates for 16 h and the treated with serially diluted test compounds at concentrations ranging from 0.125–1.25 μM. Following additional 3 days of incubation, cell proliferation was determined. Results shown are means ± SD from three experiments (n = 3). Analyses for multiple group comparisons were performed with 1-way ANOVA. * P < 0.05, ** P < 0.01 and *** P < 0.001 (compound treated vs 0.1% DMSO control).

Interestingly, all these stilbenoids (except gnetin H) were able to significantly ($p < 0.01$) enhance, by about 100–200%, the proliferation of HaCaT cells, a spontaneously immortalized human keratinocytes from normal skins (10) (Figure 4).

The immunomodulatory effect of resveratrol is not very well studied and little is known about effects of its oligomers. To explore the potential immunomodulatory activities of these stilbenoids, we isolate PBMCs from 2 normal donors and stimulated cells with 500 ng/ml of staphylococcal enterotoxin B (SEB). SEB is a superantigen because of its ability to stimulate a large fraction of T cells via interaction with T cell receptors (TCR) and class II MHC molecules (11). The simultaneous binding of SEB outside of the MHC on antigen presenting cells and to TCRs results in eliciting T cell activation and cytokine responses (12). Using Luminex beads-based multiplex cytokine immunoassays, we found that SEB significantly stimulated expression and secretion of many cytokines and chemokines involved in immune responses, inflammation, cell differentiation and growth. Among most of these cytokines, stilbenoids could clearly upregulate their production to various degrees, for example, GRO (growth-regulated oncogene) was induced by ε-viniferin (Figure 5A); IL (interleukin)-10 by ε-viniferin and resveratrol (ε-viniferin > resveratrol) (Figure 5B); IL-6 by all 5 stilbenoids except gnetin H (Figure 5C); GM-CSF (granulocyte-macrophage colony-stimulating factor) by all 5 stilbenoids (Figure 5D); and many others (Figure 5E–H). However, there were marked downregulation of some

cytokines, such as IFN-γ by all compounds except resveratrol and ε-viniferin (Figure 5J); IL-10 by gnetin H and suffruticosol B (Figure 5B), MIP (macrophage inflammatory protein)-1β by gnetin H (Figure 5E) and VEGF (vascular endothelial growth factor) by all compounds except suffruticosol C and ε-viniferin (Figure 5H).

Our immune system is highly complex self-balanced network with many subsets of cells which interact with each other and secrete various cytokines or chemokines. To further examine immunomodulatory activities of these stilbenoids on other subsets of immune cells, PBMCs were stimulated with another important stimulus, lipopolysaccharide (LPS) at 1 ng/ml. LPS is a large molecule consisting of a lipid and a polysaccharide and is found in the outer membrane of Gram-negative bacteria. LPS acts as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex on many immune cell types, especially monocytes, dendritic cells (DC), macrophages and B cells, which then promotes the secretion of pro-inflammatory cytokines, nitric oxide, and eicosanoids (13). In our experiments, the tested stilbenoids could modulate production of many anti- and pro-inflammatory cytokines and chemokines. As shown in Figure 6, 24 h of stimulation with 1 ng/ml LPS caused marked increase in production of many cytokines from PBMCs. While the stilbenoids at 0.125 μM had a weak effect on the most of these cytokines, 0.125 μM ε-viniferin could clearly upregulate the production of IL-6 (Figure 6A). Although most of these stilbenoids (except

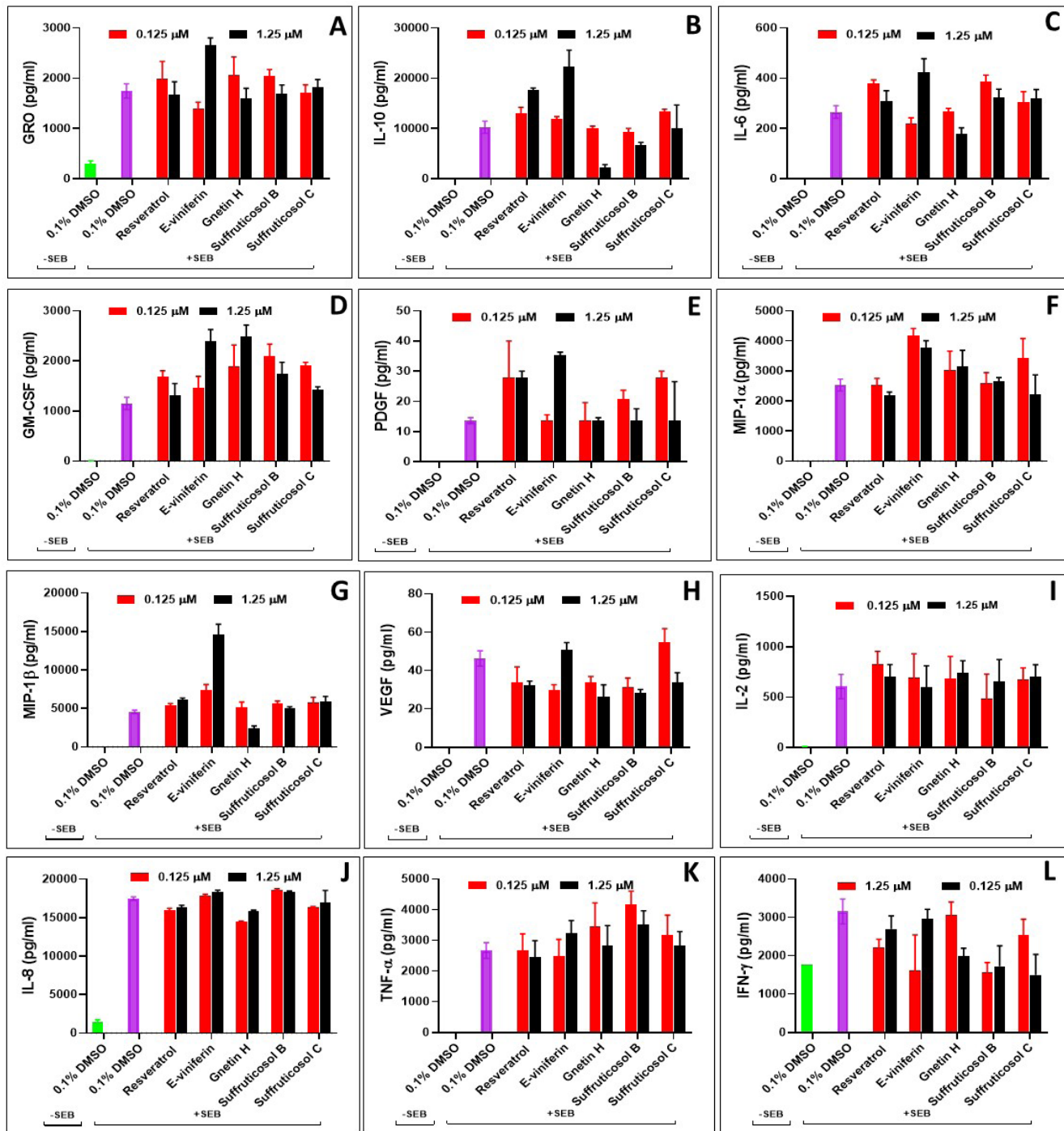


Figure 5. Effects of compounds on cytokine production by SEB induced human PBMC. Normal human PBMC cultures were pre-treated for 30 min with test compounds. The cells were then subject to a further 72-hour stimulation of 500 ng/ml SEB. Following these treatments, cell culture supernatants were harvested for the Luminex-based cytokine assay. The green bar is for unstimulated PBMC control; the purple bar is for SEB-stimulated PBMC control. Results shown are means \pm SD from two separate experiments using 2 donors (n=2).

resveratrol or gnetin H) at 12.5 μ M were found to have a general toxicity to PBMCs, 12.5 μ M resveratrol or gnetin H were able to upregulate the production of several cytokines including IP (interferon- γ inducible protein)-10, TNF- α , IL-23 and IL-4 (data not shown), suggesting that some cell subsets could be activated extensively. In contrast, suffruticosol B and

C at 1.25 μ M reduced the production of IP-10 (Figure 6B), TNF- α (Figure 6C), IL-1 α (Figure 6D), IFN- γ (Figure 6E) and IL-4 (Figure 6F). The production of IL-1 β , IL-10 and IL-23 was relatively unaffected (Figure 6G-I). Based on these cytokine data, stilbenoids could possess immunomodulatory activities on T cells, B cells, monocytes, and other immune

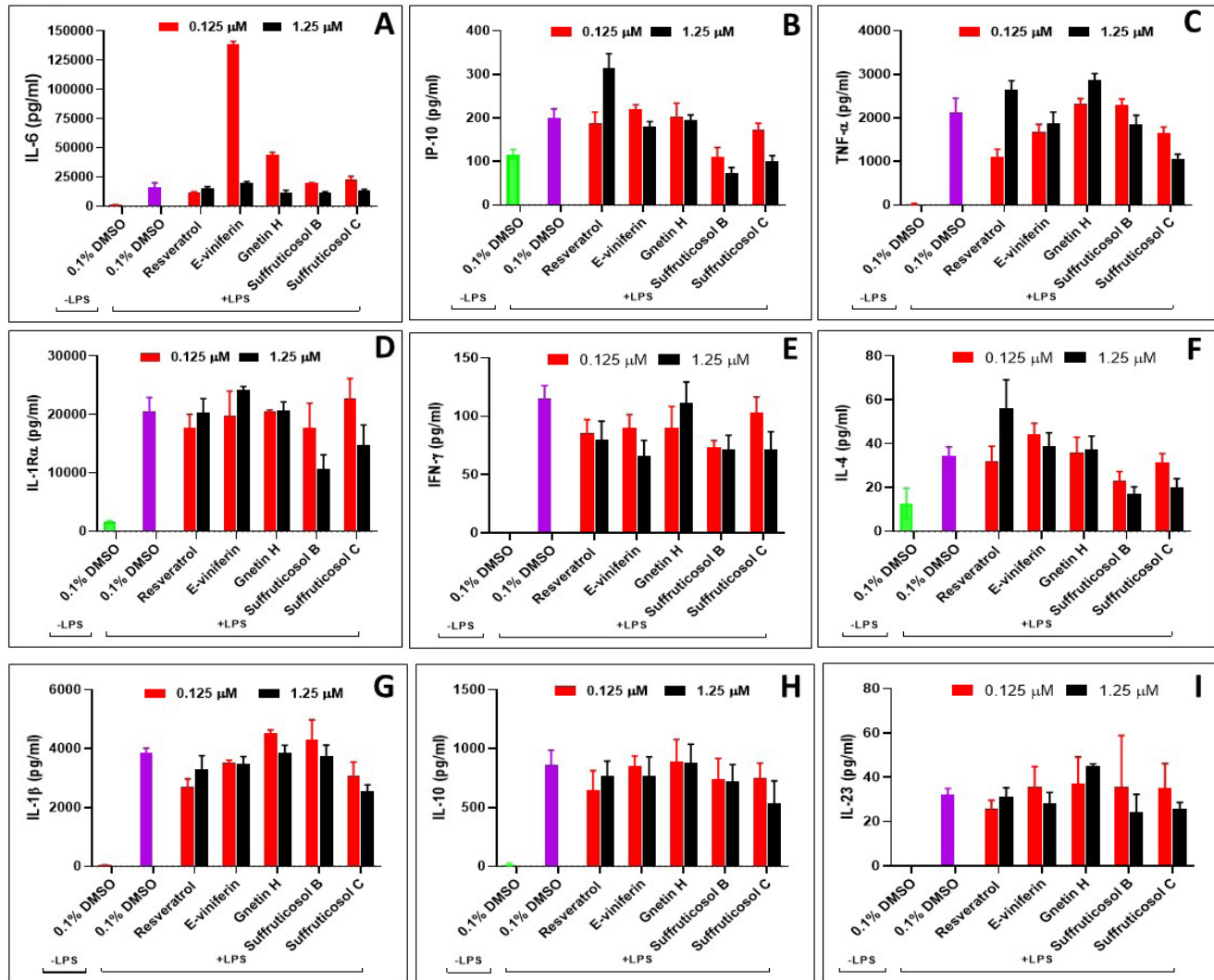


Figure 6. Effects of compounds on cytokine production by LPS induced human PBMC. Normal human PBMC cultures were pre-treated for 30 min with serially diluted test compounds. The cells were then subject to a further 24-hour stimulation of 1 ng/ml LPS. Following these treatments, cell culture supernatants were harvested for the Luminex-based cytokine assay. The green bar is for unstimulated PBMC control; the purple bar is for SEB-stimulated PBMC control. Results shown are means ± SD from two separate experiments using 2 donors (n=2).

cells. Although, it is of note, that the activities appeared not correlated with the degree of oligomerization. It definitely needs further studies to dissect which specific immune cell subsets and cytokines are consistently modulated using more donors of PBMCs or immune cells.

DISCUSSION

In 1985, Kimura, *et al* reported that resveratrol and other monomer stilbenoids could have anti-inflammatory activity by inhibiting arachidonate metabolism in rat leukocytes *in vitro* (14). In 1997, the cancer chemo-preventive activity of resveratrol through antioxidant and antimutagen effects was discovered (8). In the latest decade, more and more natural stilbenoids, including monomers and oligomers, have been isolated from various plants (15). In this study, we aimed to compare resveratrol and its oligomers (2 dimers and 2 trimers) for their cytotoxic, anti-cancer, immunomodulatory and anti-

inflammatory activities.

Our data show that resveratrol appeared to have less toxicity to normal blood cells in the presence and absence of stimuli, while dimers and trimers show more toxicity to normal PBMC. The order of cytotoxicity is: suffruticosol C (trimer) > suffruticosol B (trimer) > gnetin H (dimer) > ε-viniferin (dimer) ≈ resveratrol. It is unclear why oligomers appeared to be more cytotoxic. However, more oligomers should be investigated in order to conclude any correlation between the oligomerization degree and toxicity.

Tested at non-toxic lower concentrations, three stilbenoids were found to have some anticancer activities. Particularly, the proliferation of T98G human glioblastoma cells was clearly blocked by resveratrol, ε-viniferin and suffruticosol C. It will be interesting to test these compounds using more lines of cancer cells originated from various tissues. Our preliminary data warrant a further study to understand why T98G cells

1 were more sensitive to some of these stilbenoids than other
2 cancer cells tested, and the potential molecular mechanism
3 involved in the anticancer activity.

4
5 Zhou (16) and Park *et al.* (17) reported photoprotection
6 properties of resveratrol: resveratrol at 10 μ M significantly
7 protect HaCaT cells from apoptosis caused by UVB irradiation
8 through upregulating expression of HSP27 (heat shock
9 protein 27), increasing Bcl-2/Bax ratio, inhibiting activity of
10 caspase-3 and -8 and expression of proapoptotic proteins
11 such as p65 and Bax, and reducing production of reactive
12 oxygen species. Their data also showed that resveratrol at
13 10-100 μ M could reduce caspase-3 activity and increase
14 phosphorylation of Rb protein in HaCaT cells without UVB pre-
15 exposure. To our surprise, resveratrol and other stilbenoids
16 at as low as 0.125 μ M could actually promote proliferation of
17 HaCaT keratinocytes even without UVB stress. During cancer
18 targeted therapy, especially for non-small cell lung cancer and
19 metastatic colorectal carcinoma, serious cutaneous toxicities
20 from EGFRi (epidermal growth factor receptor inhibitors)
21 treatments are quite common, affecting 45–100% of patients
22 (18). Inhibition of keratinocyte growth and survival is one of
23 major mechanisms of EGFRi toxicity in the skin (19). Our data
24 suggest that these stilbenoids might have a potential to protect
25 keratinocytes from apoptosis to reduce the adverse effects of
26 EGFR-targeting therapies.

27
28 It has been reported that repeated doses of resveratrol (1
29 g/day for 28 days) significantly increased circulating immune
30 cells in healthy individuals, including $\gamma\delta$ T cells and regulatory
31 T cells. Resveratrol also significantly improved the growth of
32 $\gamma\delta$ T cells and regulatory T cells *in vitro* (20, 21). Our data
33 show that resveratrol and oligomers could regulate production
34 of a variety of cytokines from T cells in SEB-activated PBMCs,
35 and well as from monocytes, B cells and other immune cells
36 in LPS-stimulated PBMCs. This data suggests that resveratrol
37 oligomers, similar to resveratrol, could modulate the activity
38 of various subsets of immune cells. Our data warrant more
39 systematic studies to clearly understand resveratrol and its
40 oligomers as immunomodulatory agents due to the complexity
41 of immune cell and cytokine network.

42
43 Although our *in vitro* study results provide some
44 preliminary evidence that resveratrol and its novel oligomers
45 have anti-cancer and immunomodulatory activities, our data
46 have limitations and also prompted many questions for future
47 studies. Further studies are needed for these compounds
48 using more variety of cancer cell lines originated from human
49 tissues, and other subsets of immune cells such as natural
50 killers. It will be also interesting to examine activities for
51 immune cell-mediated cell killing of cancer cells. In order
52 to further explore the potential of these novel compounds
53 as preventive or therapeutic agents for cancers or many
54 immune-related diseases, *in vivo* studies using various animal
55 models are definitely needed to confirm the activities we
56 observed before any clinical study can be performed. As far
57 as the superiority of efficacy of these stilbenoids for human
58 wellness, more studies are needed and more factors should

be considered such as oral bioavailability, water solubility,
stability, safety and others.

MATERIALS AND METHODS

Test compounds

The five stilbenoid compounds were simultaneously purified from the dried seeds of *Paeonia suffruticosa* Andr. (*Paeoniaceae*) as described previously (12) and provided by Professor He, CN at Peking Union Medical College, Beijing, China.

PBMC isolation and stimulation

Peripheral blood from three healthy donors was obtained from BioIVT, Co. (Hicksville, NY). PBMCs were isolated by Ficoll®-Paque Premium density gradient centrifugation, following the protocol from the manufacturer (GE Healthcare, Marlborough, MA). The buoyant layer was removed and the PBMC layer collected. Following several washes in PBS to remove platelets, PBMCs were collected and incubated in RPMI-160 medium supplemented with 10% FBS (fetal bovine serum,) and 1 \times penicillin/ streptomycin/L-glutamine in a humidified incubator at 37°C with 5% CO₂. Following pre-treatment with test compounds for 1 hour, cells were either stimulated by the addition of 500 ng/ml SEB (Toxin Technology, Sarasota, FL) or 1 ng/ml LPS (Millipore Sigma, St Louis, MO) for 24 h before cell culture supernatants were collected for cytokine assays.

Cell culture of cell lines

A-172 (Glioblastoma), T98G (Glioblastoma), U-87 (Glioblastoma), and HaCaT (keratinocytes) were purchased from ATCC (ATCC®, Manassas, VA) and cultured in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μ g/ml), and L-glutamine (292 mg/ml), and maintained in a humidified incubator at 37°C with 5% CO₂. Karpas-422 and RIVA cells (human diffuse large B cell lymphoma) were purchased from DSMZ (Braunschweig, Germany) and cultured in the same complete RPMI-1640 medium as used for PBMC.

Cell proliferation assay

Cell proliferation was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), per the manufacturer's protocol. Briefly, in 96-well flat-bottom opaque-walled cell culture plates, the medium control wells containing 100 μ L /well cell culture medium without cells were used to obtain a value for background luminescence. For treatment wells, cells were seeded at 2,000 cells in 50 μ L /well of complete RPMI-1640 or DMEM medium. Following incubation overnight, cells were treated by adding 50 μ L /well of the vehicle control (0.1% DMSO) or test compounds at indicated concentrations. After cells were treated for three days at 37°C in a humidified 5% CO₂ incubator, the plates were equilibrated at room temperature for approximately 30 minutes. 100 μ L of CellTiter-Glo® Reagent was added into

each well, and then contents were mixed for two minutes on an orbital plate shaker to lyse cells. After the plates were incubated at room temperature for ten minutes, luminescent signals of each well were recorded with a Tecan M200 Plate Reader (Tecan, Switzerland). Cell proliferation in each test sample from the experiments was examined in duplicate. Compound effects on cell growth were normalized against the DMSO control after cell baseline ATP level was subtracted.

Luminex multiplex cytokine assay

After treatment of PBMCs with test compounds, 100 μ L of cell culture supernatants from each well was collected and stored at -80°C for Luminex-based multiplex assays using ProCarta cytokine assay kits (Thermo Fisher Scientific, Waltham, MA) for the following cytokines: TNF- α , IFN- γ , IL-1 β , IL-2, IL-10, GM-CSF, GRO, IL-1R α , IL-6, IL-8, IL-12, PDGF, VEGF, IP-10, IL-23, IL-4, MIP-1 α and MIP-1 β . Assays were run according to the manufacturer's protocol. Briefly, 25 μ L culture supernatants, 25 μ L assay buffer, 25 μ L antibody-coated bead mixture were added into each well in assay plates and incubated overnight at 4°C on a plate shaker. After washing beads, 25 μ L per well of biotinylated detection antibody cocktail and then 25 μ L per well streptavidin-phycoerythrin (PE) were added. Following 30 min incubation, data was collected using a Bio-Plex 200 system (Bio-Rad, Hercules, CA). A five-parameter regression analysis was used to calculate the cytokine concentrations from standard curves.

Statistical analysis

All experiments were performed in triplicate or repeated for 2-3 times ($n=2$ or 3), except as otherwise indicated. Data were graphed and analyzed using GraphPad Prism 8.4 (San Diego, CA); analyses for multiple group comparisons were performed with 1-way analysis of variance (ANOVA) followed by Dunnett's posttest. A value of $p < 0.05$ was considered significant in all analyses.

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