ABSTRACT

Title of Document: DIFFERENT EFFECTS OF RESVERATROL ON HUMAN THP-1 MONOCYTES AND THP-1 DIFFERENTIATED MACROPHAGES

Li Feng, Master, 2014

Directed By: Professor, Dr. David K. Y. Lei, Nutrition and Food Science

Resveratrol (Res), a natural polyphenol compound found in grapes and red wine has been shown to exhibit anti-tumor, anti-oxidant and anti-inflammation effects. However, the molecular targets as well as mechanisms of action contributing to the health promoting effects of Res are largely unknown. In our study, through dietary or pharmaceutical consumption levels, we investigated the effects of Res on inflammation-driven immune responses in human THP-1 monocytes and human Phorbol 12-myristate 13-acetate (PMA) differentiated THP-1-derived (P-THP-1) macrophages. Our results showed that Res induced anti-proliferation in THP-1 monocytes with S phase arrest at dietary concentrations, and, however, induced cell apoptosis and caused G0/G1 phase arrest at pharmaceutical concentrations. In addition, Res showed different effects on pro-inflammatory cytokines in different cell lines. Furthermore, similar analysis on pterostilbene and genistein revealed the possible effect was attributed to specific stilbene structure. In conclusion, resveratrol and pterostilbene appeared to play different roles of inflammatory response in THP-1 and P-THP-1 cells.

DIFFERENT EFFECTS OF RESVERATROL ON HUMAN THP-1 MONOCYTES AND THP-1 DIFFERENTIATED MACROPHAGES

By

Li Feng

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2014

Advisory Committee:

Dr. David K. Y. Lei

Dr. Thomas T. Y. Wang

Dr. Seong-Ho Lee

Acknowledgement

First and foremost, I would like to give my grateful appreciation to my advisor in University of Maryland, Dr. David Lei, and research advisor in USDA-ARS, Dr. Tom Wang for their enormous support and guidance of my graduate study and research. I would also like to thank my third member of my committee. I greatly appreciate the guidance and help of Dr. Lee. I also need to thank Dr. Norberta Schoene for helping me to perform cell cycle experiments in my project. Furthermore, I would also like to thank the senior scientists, Dr. Junjun Zhang, Dr. Kim, and my fellow lab mates, Haiqiu Huang and Quynhchi Pham, who encouraged me, supported me, and gave me help with my Master's thesis work. Lastly, I would like to thank my husband, family and friends. Their love and encouragement provided support for completing this project. I thank you all.

Table of Contents

List of Tables

List of Figures

Chapter 1 Literature review

1.1 Inflammation

1.1.1 Immunology and inflammation

Inflammation is a complicated biological process that regulates the vascular tissues' defense against harmful stimuli, such as pathogens, damaged cells, signals or irritants. In ancient Rome, Cornelius Celsus first recorded the signs of inflammation in his book "De Medicina". Four symptoms of inflammation were noted: redness and swelling with heat and pain [1]. Later on Rudolf Virchow added "loss of function" for inflammation, as well as "inherently pathological reaction", many centuries later [2]. Nowadays, it is considered as nonspecific/innate immune response, compared to adaptive immunity, which can be caused by trauma, chemical agents, thermal extremes of heat or cold, and pathogenic organisms. The symptoms of inflammation include: increased blood flow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids and cellular influx. Without inflammation, infections would go without checked, wounds would never heal, and injured tissues would remain damaged.

Acute and chronic inflammation

Inflammation includes: acute and chronic one. When facing exogenous pathogens, acute inflammation is the first response of our body. The inflammatory response has a cascade of biochemical events in vascular system and the injured tissues, which may last a few minutes to a few days. After the injured site is healed, anti-inflammatory cytokines

work to end the inflammation. Nevertheless, chronic inflammation leads to an endless inflammation and tissue destruction resulted from enhanced expression of pro-inflammatory cytokines [3]. One difference between acute inflammation and chronic inflammation is that injured tissues are infiltrated by mainly neutrophils within 24 to 48 hours in acute inflammation, whereas chronic inflammation is more associated with monocyte differentiated macrophages and lymphocytes [4].

Innate and adaptive immune response

According to the book of "Kuby immunology" written by Thomas J. Kindt [5], there are two kinds of immune response: innate and adaptive. To protect our body from outside invasion by pathogenic microbes and toxins, our immune system has evolved well to defend against them. Different reaction and duration time, reaction cell types, and specificity for variable types of pathogens have been noted between innate and adaptive immune responses.

The innate immune system is the first line of defense to protect the host from pathogens, which is mediated by phagocytes (monocytes, macrophages and dendritic cells), which reacts in a couple minutes or hours. The main components of the innate immune system are physical epithelial barriers (e.g., skin, anti-microbial compounds), phagocytic leukocytes, dendritic cells and natural killers (NK). Antigen-presenting cells (APCs) like macrophages and dendritic cells from innate immune system then display a fragment of the antigen from engulfed pathogens, which will initiate the adaptive immune response. Compared with the innate immune response, adaptive immune response is mounted after several days of specific antigenic challenges. If innate

immunity is like a key to open a building, adaptive immunity is like a key to open a specific door. The adaptive immune response can be further divided into two subgroups: humoral immunity (B lymphocytes) and cell-mediated immunity (T lymphocytes). B-cells are produced and matured in bone marrow. When they leave the bone marrow, each B cell expresses a specific antigen-binding receptor on the membrane, which can recognize antigen alone. The principal functions of B-cells are to make antibodies against antigens, to perform the role of APCs, and to develop into memory B-cells after activation by antigen interaction. Unlike B lymphocytes, T lymphocytes are produced in the bone marrow, and then migrate to thymus gland to mature. T-cells response to major histocompatibility complex (MHC) I or II to perform either cell-killing or secreting cytokines functions, respectively. T-helper (T_H) cells display CD4 and are activated by recognizing and interacting with an antigen-MHC class II molecule complex, which becomes an effector cell to secret various cytokines for the activation of other immune cells. On the other hand, T cytotoxic (T_C) cells, another type of T cells, display CD8, which can recognize antigen-MHC class I molecule complex. Once initiated, T_C cells proliferate and differentiate into cytotoxic T lymphocytes (CTLs). The CTLs have a vital function in monitoring the cells of the body and eliminating any that display antigen, such as virus-infected cells, tumor cells, and cells of a foreign tissue graft [6].

Pathogen recognition and TLR signaling pathway

Innate immune system is capable of mounting a non-specific response when compared with adaptive immune system. However, innate immunity is not completely

nonspecific, rather it can recognize conserved limited number of germline-encoded pattern-recognition receptors (PRRs) [7]. PRPs are able to: 1) recognize pathogen-associated molecular patterns (PAMPs), which are small molecular motifs essential and conserved for the survival of the microorganism; 2) detect the pathogens and express constitutively in the host all through the life cycle; 3) express on all the cells with certain type, which are independent of immunologic memory.

PRRs are found as membrane-bound, cytoplasmic and secreted forms. The Toll-like receptor (TLR) family, one of PRRs, plays an important role in innate immunity. TLRs are trans-membrane proteins, which can recognize extracellular or endosome PAMPs and initiate cascade-signaling pathways. To date, there are 10 members of TLRs found in humans and 13 in mice. TLRs are expressed on macrophages, dendritic cells, B cells, and some types of T cells, fibroblasts and epithelial cells. Several different kinds of TLRs can recognize related PAMPs. For example, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TLR11 can recognize bacteria; TLR2, TLR4, TLR6 can recognize fungus; TLR2, TLR4, TLR9, TLR11 can recognize parasites; and TLR2, TLR3, TLR4, TLR7, TLR8, TLR9 can recognize viruses. Interestingly, the same TLR can also recognize several uncommon ligands. TLR4 plays important role in immune system in that it can recognize diversified ligands such as lipopolysaccharide (LPS) from gram-negative bacteria, mannan of fungus, glycoinositol phospholipids from parasite, envelop proteins of respiratory syncytial virus, heat-shock proteins and fibrinogen, which have different structural features [7]. TLRs then activate downstream signaling pathway to induce inflammatory cytokines, like TNF-α, IL-1β, IL-6, IL-12 and other effect mediators to work against different kinds of pathogens [8].

Cellular mediators of inflammation

Cytokines are part of a category of signaling molecules (non-antibody proteins) that mediate and regulate cell growth, immunity, inflammation, hematopoiesis, tissue repair, and many other cellular processes forming a cytokine network.

They were mediators and regulators among immune response, but now many cytokines are found to either be produced by non-immune cells or induce effects on non-immune cells. The cytokines released by a certain type of cells and then bind to specific receptors on target cells, where cellular response may take several hours to produce new mRNA and synthesis protein. The target cells can be divided into three groups: autocrine (target is the same cell), paracrine (nearby cells), and endocrine (distant cells running through the circulation).

The term of cytokine is a general name for those proteins, whereas it can be categorized in different groups based on different function and purpose. Monokines are those cytokines produced from monocytes and macrophages, and lymphokines come from lymphocytes.

Interleukins are a group of proteins secreted primary from helper T lymphocytes, monocytes, macrophages and endothelial cells. Interleukin1 to 36 act significantly as signaling molecules through cells in immune systems to regulate cell growth, differentiation and motility of T, B and hematopoietic cells, whereas excess or deficient interleukins may be involved in autoimmune diseases or immune deficiency, respectively [9]. Interleukins are released quickly out from inside of the cells, and travel to target cells for further proliferation and activation or regulation, which is a rapid cascade signaling

process to initiate other cell's behavior. IL-1 is secreted from monocytes, macrophages, B cells and DCs, and targets on T-helper cells for co-stimulation, B-cells for proliferation, and NK cells for activation. Small amount of IL-1 induce acute inflammation, whereas large amount may give rise to fever. IL-6 is a key interleukin expressed in both acute inflammation and chronic inflammation. In acute inflammation, IL-6 is secreted from the site of inflammation to mediate the reaction. When in chronic inflammation, IL-6 plays key role to attract mononuclear cell accumulation through MCP-1 secretion by activating gp130 in which the transition of acute inflammation to chronic inflammation is from neutrophils reaction to monocyte recruitment [10].

TNF refers to a family of tumor necrosis factors, which can cause cell apoptosis with nineteen cytokines included [11]. The two main TNF family cytokines are TNF-α and TNF-β.TNF-α is mainly secreted by monocytes or macrophages and functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine acts as pro-inflammatory cytokine, which is involved in the regulation of cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. Like IL-6, TNF-α is an endogenous pyrogen causing fever and other inflammation symptoms, which is also implicated in tumor regression, septic shock, and cachexia [12]. On the other hand, TNF-β, derived from Th1 type T-cells, secretes for the induction of change of surface adhesion molecules on certain cells to allow them to bind to phagocytes during inflammation [13].

Chemokines are a group of signaling cytokines of small sizes (8-10 kilo Daltons) featuring in recruiting leukocytes to places of inflammation. They mediate immune cells' trafficking. Their name is formulated because they induce chemotaxis to target cells, so they are also called chemotactic chemokines. There are four cysteine residues forming

their conservative three-dimensional structure. Some chemokines are pro-inflammatory and they can be induced during an immune response to recruit cells of the immune system to a site of infection, whereas some other chemokines can control the migration of cells during normal processes of tissue maintenance or development.

Table 1. Cytokines and functions [14**]**

Cytokines	Cell source	Response to	Functions
IL-1β	Macrophages	T-cells, B-cells, Endothelialcells	1. T-cell activation 2. Endogenous pyrogen 3. B-cell maturation and proliferation 4. Fibroblast growth factor activity
IL-2	Th1 cells	CD8 T-cells, B-cells, NK	1. T and B-cell proliferation 2. Stimulate monocytes, lymphokine-activated killer cells, natural killer cells, and glioma cells
IL-3	Activated Th cells	Bone marrow stem cells	Stimulates progenitor cells of most cell lineages in bone marrow
IL-4	Th2 cells	B-cells	1. B-cell growth 2. Isotype switching to IgE and mast cell proliferation 3. Th2 induction and MHC-II induction on B-cells 4. T-cell growth factor
IL-5	Th2 cells	B-cells, Eosinophils	1. B-cell differentiation 2. Main regulator of eosinopoiesis, eosinophil maturation and activation
IL-6	Th cells, Macrophages	B-cells	1. Inflammation and maturation of B-cells 2. Endogenous pyrogen
IL-8	Macrophages	Neutrophils	1. CXC chemokine family 2. Attracts neutrophils, basophils, and T-cells, but not monocytes 3. Neutrophil activation
IL-10	Th2 cells, Monocytes	Th1, B-cells, Macrophages	1. Suppress Th1 cells, MHC class-II Ags 2. Co-stimulatory molecules on macrophages 3. Enhance B-cell survival, proliferation, and antibody production 4. Block NF-kappa B activity, and involve in the regulation of the JAK-STAT signaling

			pathway
IL-12	APCs	Th1	Induces Th1 cells
IL-15	Non-T-cell w/MHC I/II cells	T-cells and NK	1. Activates Th and NK 2. Stimulation by IL-15 requires interaction of IL-15 with components of IL-2R, including IL-2R beta and probably IL-2R gamma but not IL-2R alpha
IFN-γ	Th1 cells	Macrophages, B-cells, T-cells	1. Inhibits IL-4 effects 2. Activates macrophages-promotes cellular response 3. Inhibition of Th2 responses 4. Terminal plasma cell differentiation
TGF-β	Th1 cells	B-cells, T-cells, Macrophages	1. IgA class switching 2. Inhibits IL-4 3. Is chemotactic for T-cells and Macrophages
TNF-α	Th1 cells, Macrophages	Neutrophils, T-cells, Endothelialcells	1. Mediator of Septic shock in high concentration 2. T-cell activation 3. Pyrogen 4. Endothelial adhesion molecule expression
GM-CSF	T-cells, Macrophages	Macrophages, Neutrophils	Enhances Macrophages and neutrophil activity
MIP-1	Macrophages	T-cells, monocytes	1. Chemotactic factor that attracts T-cells and monocytes, but not neutrophils, eosinophils, or B-cells 2. CC type chemo
MCP-1	Monocytes, endothelial cells	T-cells, Macrophages	1. Monocyte chemotactic protein 2. CC type chemo
IP-10 and MIG	Macrophages	T-cells	CXC chemokines that affect T-cell recruitment
IL, interleukin; Ags, antigens; Th, helper T cells; APCs, antigen-presenting cells; IFN, interferon; NK, natural killer; IP, interferon- γ inducible protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP, monocyte chemotactic protein; MIG, monokine induced by IFN- γ; MIP, macrophage inflammation protein; TNF, tumor necrosis factor;			

1.2 Resveratrol and its protective effect

1.2.1 Resveratrol

Resveratrol (3, 5, 4' –trihydroxy-*trans*-stilbene) is a phytoalexin found in a variety of foods (e.g., grapes, peanuts, red wine, and berries) in response to environmental stress, injury, UV irradiation, and fungal infection. Resveratrol (Res) has been shown to exert anti-oxidant, anti-cancer and anti-inflammatory effects [15, 16, 17]. Res is also known as 3, 4', 5-stilbenetriol in *cis* and *trans* isometric forms (trans-isoform is more widely investigated).

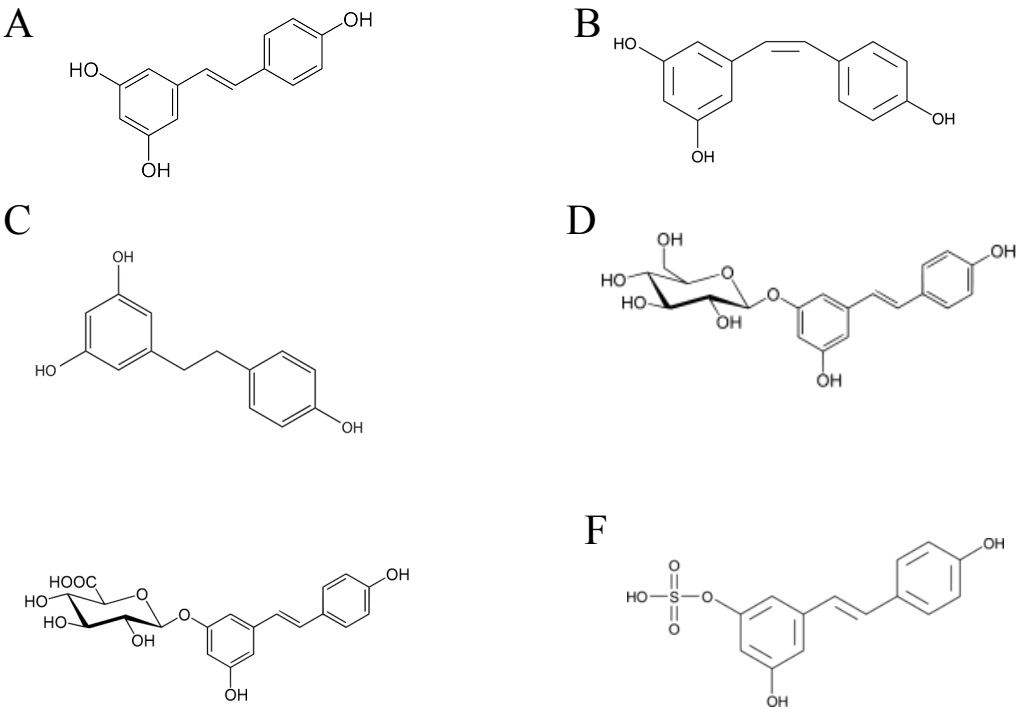


Figure 1. Chemical structure of (A) *trans*-resveratrol, (B) *cis*-resveratrol, (C) dihydroresveratrol, (D) *trans*-piceid, (E) resveratrol-O-glucuronide, (F) resveratrol-sulfate

Furthermore, piceid was considered as a resveratrol alternative because *trans*-piceid can be enzymatically hydrolyzed to *trans*-Res in the colon or inside enterocytes (the same for *cis*-piceid which can be converted to *cis*-Res) [18]. Piceid, is named as resveratrol 3-O-β-D-glucoside, and is the main component of the Polygonumcuspidatum root, used in Chinese and Japanese traditional medicine for treating some chronic inflammation disease, and also naturally exists in wine and fruit juice. Interestingly, there is higher level of piceid than resveratrol in grape juice. The average concentrations were 3.38 mg/L for *trans*-piceid, 0.79 mg/L for *cis*-piceid, 0.50 mg/L for *trans*-resveratrol, and 0.06 mg/L for *cis*-resveratrol in red grape juice [31]. Through metabolism in humans, around 60% *trans*-piceid may be converted to *trans*-resveratrol in the body [31]. Hence, drinking wine is similar to drinking grape juice on the effect of resveratrol manner.

1.2.2 Food source

Resveratrol comes from peanuts, grape skins, strawberry, blueberry, mulberry, cocoa powder, as well as Japanese knotweed. From Dr. Baur's review on resveratrol [16], higher contents of *trans*-res were listed here: red wines with 0.1-14.3 mg/L *trans*-Res; white wines with less than 0.1-2.1 mg/L *trans*-Res; red grape juice with 0.5 mg/mL; cranberry raw juice with 0.2 mg/mL; boiled peanuts with 5.1 μg/g; dark chocolate with 2 μg/g [19]; and rhubarb dry root with 3.9 mg/g (Chinese medicine). Dr. Stervbo reported the highest level of *trans*-Res in wines: Pinot Noir, St. Laurent, Marzemino, Merlot, and Blaufrankisch with concentrations of 3.6 ± 2.9 mg/l (15.9 ±12.5 μM), 3.2 ± 1.8 mg/l (14.0 ± 8.1 μM), 3.0 ± 2.1 mg/l (13.1 ± 9.2 μM), 2.8 ± 2.6 mg/l (12.5 ± 11.5 μM), 2.6 ± 1.3 mg/l (11.3 ± 5.5 μM), respectively [20]. Resveratrol is a natural phytoalexin, a defender of the plant in response to injury (trauma, UV light and infection by fungi). In

cooler climate (fungi infection) and zone of equator, high concentration of resveratrol is produced in grapes [21, 22, 23]. Besides Res sources from food or wines, supplements (125 – 1000 mg/capsule) of Res are also available on the market from different companies.

1.2.3 Bioavailability and metabolism of resveratrol

In recent years, a large number of researches reported the protective effect of resveratrol on cancer, heart disease, aging and Alzheimer's disease [24, 25, 26, 33, 27]. However, higher dosage of Res (used in in-vitro studies) is hard to be reached in humans by dietary intake. In humans, resveratrol is highly absorbed, rapidly metabolized, mainly into sulfates and glucuronide–conjugates, by the stomach and liver, which are eliminated in urine.

Resveratrol is metabolized in the intestine and then in the liver to sulphated and glucuronidated forms that are exported to target organs. With the development of the testing method on resveratrol metabolites, more metabolites can be detected from plasma, urine and feces. However, the quantity of metabolites differs from human objects. In early studies, *trans*-res glucuronides and sulfates (figure 1) were measured by using enzymatic method in which biding glucuronidase or arylsulfatase [28, 29, 30]. Recently, HPLC MS/MS improves the detection. Glucuronides and sulfates conjugates are the most frequently detected metabolites from plasma or urine, whereas other metabolites include dihydroresveratrol, disulfate, diglucuronides and glucuronide-sulfate [31, 32, 33]. Correspondingly, metabolites of 160 mg piceid administration are similar to 100 mg free resveratrol [31].

Since resveratrol has high absorption rate but very low bioavailability, which

means that Res is also rapidly metabolized to conjugates in liver after consuming from mouth, resulting in low or no detectable level of resveratrol in plasma [34], whether res-conjugates has any disease-protective effect like Res should be taken into consideration. In 2012, Carolina and Juan examined the three main metabolites of resveratrol, resveratrol-3-O-sulphate, resveratrol-3-O-glucuronide and resveratrol-4'-O-glucuronide, on the effect of cell growth inhibition, the cell cycle and apoptosis using human adenocarcinoma cell line (Caco-2 cell) cultures. Compared to pure resveratrol, they found that Res metabolites have similar antioxidant activity, inhibition on cell growth in a concentration-dependent manner with an increase in G0/G1 phase as well as the induction of apoptosis [35].

However, in 2011, Johnson et al., reported that piperine (derived from black pepper) can enhance the bioavailability of resveratrol in mice [36]. Twenty-four mice received a single dose of resveratrol (100 mg/kg via oral gavage) while the other 24 mice received combination resveratrol (100 mg/kg via oral gavage) plus piperine (10 mg/kg via oral gavage). Concentration of resveratrol and resveratrol-3-O-β-D-glucuronide were tested by LC/MS from blood samples with 8 time points from fifteen minutes to 2 days. And pharmacokinetic parameters were determined by area under the concentration-curve (AUC). The researchers found that the AUC was enhanced to 229% (from 5046 ng/mL h to 11584 ng/mL h) and the maximum serum concentration was increased to 1544% (peak from 2277 ng/mL to 35169 ng/mL at 0.25 h) with the addition of piperine [36]. This research is meaningful for the application of dietary resveratrol in future human clinical trail that blood concentration of Res can reach the pharmacological effect from the study in vivo.

1.2.4 Toxicity

With short period time of oral resveratrol intake, animal and human studies showed Res appeared to be well tolerated without side effects, except for the dose of 4 g and 2.5 or 5 g per day for two human studies [37,38]. In 2010, Dr. Le Porte reported a study [37] based on eight healthy human subjects who were given 2 g Res twice per day, that they had uncomfortable symptoms including mild episodic diarrhea/loose stool, rash and headache at the beginning of the treatment. From Dr. Elliott's study [38] on healthy volunteers with treatment of 2.5 or 5 g Res daily for 28 days, the report indicated, "Adverse events were generally mild in nature and reversible". These two human studies implicate that high doses of Res (2.5 g and above daily) may induce mild side effects in some of healthy subjects at the beginning and these side effect symptoms may become less severe after a period of time. Indeed, such hypothesis still needs more evidence to support that it is true. From one rat study [39], the safe or toxic levels were evaluated. Both male and female rats were gavaged with 0, 300, 1000, and 3000 mg *trans*-Res/kg body weight (BW)/day for 28 days. Most of the adverse events occurred in the rats administered 3000 mg/kg BW/day. Two male rats were found dead in the 3000 mg/kg BW/day group. The toxic effects of reduced body weight increased clinical toxicity, renal lesions, and increased incidence of severe nephropathy were observed in the 3000 mg/kg BW/day group [39]. From human studies, there is no valid strong evidence indicating long-term effect of toxicity in taking resveratrol, so further study should be performed.

1.2.5 French paradox

French paradox is the low prevalence of coronary heart disease (CHD) associated with high intake of dietary cholesterol and saturated fat [40], which is opposite from what we have heard "high-fat diet increases CHD rate". An epidemiological study on coronary heart disease (CHD) mortality in European reported very low CHD mortality in the regions of Lille (northern France), Strasbourg (south western France) and Toulouse (south western France), as compared with other European regions [41]. The WHO dietary recommendation for saturated fat is less than 10% of total energy, but French people consume 15% energy from saturated fat. Thus, they should be exposed to high risk of CHD but they are not [42].

Scientists then worked hard to elucidate the French paradox. Several hypotheses had been proposed and debates began to focus on alcohol consumption especially red wine, which is the common identifiable element. A European study [43] reported that the wine consumption could decrease 24-31% for all causes of mortality. The dominant theory is that the polyphenols can have anti-oxidative function on LDL and thus prevent atherogenesis and heart disease [44]. In vitro, 3.8 or 10 μmol/L of polyphenols derived from red wine inhibited LDL peroxidation by 60 and 98%, respectively [45]. Also, mild-to-moderate wine drinking habit can reduce platelet and monocyte adhesion, a variety of cancers, and Alzheimer diseases.

1.3 Hypothesis and objectives:

To date, there has been little research investigating the effect of resveratrol, with consumption attainable concentrations, on the inflammation effect on human cells. In our study, we tested the hypothesis that whether resveratrol, at dietary and pharmacological concentrations, is safe for the dosage? Has anti-inflammation effect in our cell lines? And has any structure specificity?

We chose two human immune related cell models, human THP-1 monocytes, and PMA differentiated human THP-1 macrophages to address our objectives as below:

1. Is the dosage from dietary to pharmacological level of Res safe for THP-1 or P-THP-1 cells? If not, how toxic is it?

2. By testing cell cycle and cell proliferation related genes on THP-1 monocytes treated with Res, how does Res regulate cell proliferation and exert cancer prevention effect on THP-1 monocytes?

3. How does Res modify pro-inflammatory cytokines in THP-1 and P-THP-1 cells? Does it exert induction or inhibition effect on inflammation?

4. Does Res (with basic stilbene structure) exhibit any structure specific effect?

Chapter 2 Effect of resveratrol in THP-1

2.1 Introduction:

2.1.1 Resveratrol

Resveratrol (3, 5, 4' –trihydroxy-*trans*-stilbene) is a phytoalexin found in a variety of foods (e.g., grapes, peanuts, red wine, and berries). Resveratrol (Res) level is elevated in response to environmental stress, injury, UV irradiation, and fungal infection. Res has been shown to exert anti-oxidant, anti-cancer and anti-inflammatory effects [15, 16, 17]. Res is also known as 3, 4', 5-stilbenetriol in *cis* and *trans* isometric forms (*trans*-isoform is more widely investigated).

2.1.2 Cancer prevention by resveratrol

The CDC website [46] presented the number of deaths from 10 leading causes in the United States during 2010, according to the National Vital Statistics System. In 2010, a total of 2,468,435 deaths occurred in the United States. The first two leading causes of death, heart diseases (597,689 deaths) and cancer (574,743), accounted for nearly 50% of all deaths. Around 30 – 40 % of cancer is directly linked to the diet we eat. Right now, besides genetic risk of cancer, with more incidences of environment pollution, radiation exposure, stress and imbalance hormones, chronic inflammation, unhealthy diet and physical inactivity, UV light, and other carcinogens, people have higher chance to develop cancer [47]. Cancer development starts with initiation, and then goes through promotion, progression and metastasis. Among the four stages of cancer development, only stage I refers to benign tumor, whereas stage II – IV belong to malignant cancer.

Here what we talk about cancer is from stage II – IV, not including benign tumor. In stage I, a benign tumor, non-cancerous, has a slower growth rate than malignant tumor, which is surrounded by an outer cover (fibrous sheath of connective tissue) or remains with the epithelium to maintain cohesive and remain well differentiated. On the other hand, malignant neoplasm has such type of irregular shape, rapid growth, not encapsulated, infiltrating/metastasis ability, difficulty to be removed, tend to recur after removal and life-treating [48, 49].

Cell cycle is a highly ordered process with one-direction when cells duplicate. When cells undergo the process of cell cycle, they need to transit through G1, S, G2 and M phases with two kinds of circuits-intrinsic mechanisms (act in each cell cycle to order events) and extrinsic mechanisms (act when a defect is detected) [50]. Normal regulation of cell cycle for cell proliferation is essential in eukaryotic cells, which is especially important for humans to maintain normal cells growing because any uncontrolled cell proliferation under unregulated cell cycle leads to cancer eventually. Thus, cell cycle regulation is like a protective umbrella to inhibit abnormal cell proliferation. Generally, when a cell needs to proliferate, it enters in G1 phase first, and then S, G2 and M phase to complete this process. If the DNA is damaged or mutated or the process is not properly going well, checkpoints (molecular surveillance system) from each phase have to stop the process, which is called “arrest”. G1 checkpoint, also called R-point (restriction point), is the first checkpoint, which is located at the end of G1 phase. In G1 cells, cells need to grow cell size, double organelles and synthesize proteins. If cells pass the G1 checkpoint, they then move to S phase. If DNA is damaged, cells are either arrested in G1 phase before entering into S phase, or retreat back into resting state - G0 phase. This checkpoint

is critical for the cell in making a right decision in the cell cycle. Thus, R point is a very important molecular target for cancer prevention and therapy. After finishing DNA synthesis and chromosome duplication in S phase, cells enter G2 phase, where cell prepares for mitosis. G2/M check point is located at the end of G2 phase, which monitors whether cells are normal or not to begin mitosis, and also to provide an opportunity to stall the proliferation or repair of DNA damaged cells. The last checkpoint is called spindle checkpoint located in M phase to maintain that all the chromosomes should align at the mitotic plate and be under bipolar tension [51, 52]. If mutations happen on critical genes of these checkpoints, then abnormal cells will be allow to proliferate without control and develop cancer finally.

In recent years, Res was found to be a good candidate for chemotherapy and chemoprevention, by exerting an inhibition on cancer cell proliferation and induction of apoptosis. Res is involved in intracellular signaling pathway to keep homeostasis of cell growth and differentiation, which includes various cyclins, cyclin-dependent kinases (CDk), CDk inhibitors, and checkpoint kinases (Chk) [53]. From the study of cells in culture, tissue and xenografted animals treated with Res, Res was found to targeted several different signals by either down regulating cyclin-CDks and/or up regulating CDk inhibitors on inhibition of cell proliferation [54]. By treating promyelocitic cell line HL-60 with Res, Fulvio*et al*. (1998) found that a complete arrest of proliferation in the S phase by at least 30 μM Res [55]. Similarly, in human histiocytic lymphoma U937 cell lines, Res was found to induce arrest in the S phase at low concentration (30-60 μM), whereas higher concentration didn't have same S phase arrest [56]. Both reports showed increase in cyclin A and E, and the cell cycle arrest reversible [55, 56].

On the anti-leukemia effect of resveratrol, Res exerts growth inhibition, induces apoptosis and blocks in S phase progression by being mostly dependent on the concentration of Res [57]. In 2002, Andrew *et al*., reported that apoptosis with IC50 values of 70-150 μM in five human cancer cell lines (MCF7, SW480, HCE7, Seg-1 and HL60) [58]. Similarly, Helene *et al*., (2002) reported that apoptosis was induced in their Res treated lymphoid and myeloid leukemia cells with IC50 5-43 μM [59]. From several different studies on THP-1 cells, CEM-C7H2 and Jurkart cells treated by Res, the Res-induced apoptosis was showed to be independent of Fas/FasL pathway [60, 61]. In contrast, apoptosis was undetected in Res treated HL60 cells [62, 63]. Furthermore, Res can induce mitochondrial release of cytochrome c in THP-1 cells followed by the activation of caspase-9 and downstream caspases [60]. Such evidence suggests that Res can induce apoptosis through Fas/FasL –independent pathway on specific leukemia cell types, whereas Res induces apoptosis in THP-1 cells through the mitochondrial pathway.

2.1.3 Hypothesis:

THP-1 monocytes resemble human monocytes in many biological properties, like morphological aspect and cytokine secretion. Also THP-1 is a p53 mutant cancer cell line, and is a good model to study the effect of Res on cancer prevention. In our study, we investigated the effect of Res (0-25 μM) in THP-1 cells to test its toxicity on cell viability, cell cycle, cell proliferation related genes expression and pro-inflammatory cytokines productions from THP-1 monocytes with or without LPS or PAM stimulation.

2.2 Materials and methods:

2.2.1 Material and reagents:

Cell culture media, DMEM (Cat #11,995) and RPMI 1,640 (Cat #11,875), were purchased from GIBCO (Grand Island, NY, USA). Lipopolysaccharides from *Escherichia coli* 0111:B4, resveratrol (Res), pterolstilbene (Ptes), genistein (Gen) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Strata Script First Strand Complementary DNA Synthesis kit was purchased from Stratagene (Santa Clara, CA, USA). TaqMan Universal PCR Master Mix for Real-time PCR was obtained from Applied Biosystems (Carlsbad, CA, USA).

Table 2. Material information

Name	Company	Address
Cell culture media, DMEM (Cat #11,995) **RPMI 1,640 (Cat #11,875)**	GIBCO	Grand Island, NY, USA
Lipopolysaccharides from *Escherichia coli* 0111:B4 **resveratrol** **pterolstilbene** **genistein** **Phorbol 12-myristate 13-acetate (PMA)**	Sigma-Aldrich	St. Louis, MO, USA
Pam3CSK4	InvivoGen	San Diego, CA, USA
AffinityScript Multi Temperature cDNA Synthesis Kit	Agilent Technologies	Santa Clara CA , USA
TaqMan Universal PCR Master Mix for Real-time PCR **TaqMan Assay-on-demand Gene Expression Assays:** **IL-1β (Hs01555410_m1)** **IL-6 (Hs00985639_m1)** **IL-8 (Hs00174103_m1)** **COX-2-PTGS2 (Hs00153133_m1)** **MCP-1-CCL2 (Hs00234140_m1)** **TNF-α (Hs00174128_m1)** **CYP1A1 (Hs01054797_g1)** **CYP1B1 (Hs02382916_s1)** **P21-CDKN1A (Hs00355782_m1)** **P27-CDKN1B (Hs01597588_m1)** **GAPDH (Hs02758991_g1)**	Applied Biosystems	Carlsbad, CA, USA
Human IL-6 ELISA kit II (Cat #550799)	BD Biosciences	San Jose, CA, USA

2.2.2 Cells and cell growth conditions:

THP-1 cells:

Human THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1,640 media, which contained 10% fetal bovine serum (FBS) and 1% amphotericin B/streptomycin/penicillin, hereinafter referred as the complete medium (CM). Cells were incubated at 37 °C in humidified air with 5% CO_2 in ambient air. Prior to experiments, THP-1 cells were grown in the medium for 24 hours for adaption. After 24 h, the medium was removed and replaced by fresh CM with different concentrations of resveratrol.

2.2.3 Evaluation of cell viability by Trypan Blue staining in THP-1 cells

THP-1 monocytes (1×10^5 cells/mL, 2mL) were plated in 6-well plates (Costar; Corning Incorporated, Corning, NY, USA) for 24 hours and then treated with (1, 5, 10, 25 μM) or without resveratol (control, DMSO) for 0, 24, 48 hours. Total cell count for each sample was measured with Trypan Blue staining.

2.2.4 Cell cycle analysis/FACS analysis

THP-1 monocytes (1×10^5 cells/mL, 10mL) were plated in 75-cm^2 flasks overnight and then treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. Cells were washed with PBS three times in 50-mL polypropylene tubes (BD Biosciences, Bedford, MA, USA), and then fixed in 15 mL 70% chilled ethanol overnight at 4 °C.

DNA content of the cells was determined by flow cytometry using a FACScalibur cytometer (Becton Dickinson, San Jose, CA). Washed cells were fixed in ethanol and

stained for DNA content using propidium iodide. Flow cytometric data files were collected and analyzed by using the CELLQuest program (Becton Dickinson). A total of 10,000 cell events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software (version 3.0, Verity Software House, Inc., Topsham, ME). Calibration standards (LinearFlow Green and DNA QC Particle Kit) for verification of instrument performance were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively [64, 65].

2.2.5 Total RNA isolation, cDNA synthesis, and gene expression analysis using real-time PCR

To determine the mRNA expression level in THP-1, such mRNA expression was measured by real-time PCR. THP-1 cells were cultured in 6-well plates, treated with control (DMSO), or resveratrol (1, 5, 10, 25 μM) for 48 hours. Thereafter, LPS (10 ng/mL) or Pam3CSK4 (500 ng/mL) were added for another 2 hours. Cells were harvested and washed with PBS three times and then total RNA were isolated following TRIzol protocol (Life Technologies, Grand Island, NY, USA). In brief, cells were broken in 1 mL TRIzol for 5 minutes. The aqueous phase, containing mRNA, was collected by centrifuging step, after 200 μL chloroform were added in each EP tube. Isopropanol was added for precipitating total RNA overnight at 4 °C. RNA was washed with 75% ethanol twice, and then a 0.2 μg/μL RNA solution was prepared.

One μg of total RNA was used to reverse transcribe cDNA by using AffinityScript Multi Temperature cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA). Real-time PCR amplification of mRNA levels was performed in the ABI Prism 7900HT Sequence Detection System by using TaqMan universal PCR Master

Mix (Life Technologies, Grand Island, NY, USA) according to manufacturer's protocol. Five μL of cDNA were used for real-time PCR. The TaqMan gene expression assay was used to detect IL-1β, IL-6, IL-8, COX-2, MCP-1, TNF-α, P21 and P27. The mRNA levels were normalized to an internal control, GAPDH mRNA.

2.2.6 p21 protein determination by using flow cytometry

THP-1 Cells were re-suspended in 300 mL of RPMI medium. Formaldehyde (16%) was added to give a final volume of 1.5% and cells were fixed for 15 min at room temperature followed by centrifugation. Pellets were re-suspended by vortexing in 50 mL RPMI media. Tubes were chilled and then 1 mL of ice-cold methanol was added while again vortexing to prevent cell clumping. Samples were capped tightly and stored at -20 °C until analysis by flow cytometry [66,67].

2.2.7 Statistics:

Data were analyzed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and are presented as means ± SE. A one-way ANOVA followed by post hoc analysis. The Tukey test was used to determine the statistical significance among treatments, $p < 0.05$.

2.3 Results:

2.3.1 Effect of resveratrol on the growth of THP-1 monocytes

The highest growth rate of THP-1 monocytes was observed with the DMSO controls. A similar growth rate was observed with the resveratrol (Res) 1 μM treated cells. A dose dependent inhibition of THP-1 monocyte growth was seen with higher doses of resveratrol (Figure 2A). Treatment with Res 25 μM was associated with minimal cell growth at 24 hours and cell death at 48 hours. Cells treated with Res 10 and 25 μM had a 36.5% and 69.04%, respectively, reduction in growth compared to controls at 48 hours. Res 25 μM was associated with a loss of 44.5% of cells from the initial plating. The half maximal inhibitory concentration (IC50) of Res for THP-1 cells was 17 μM (Figure 2B).

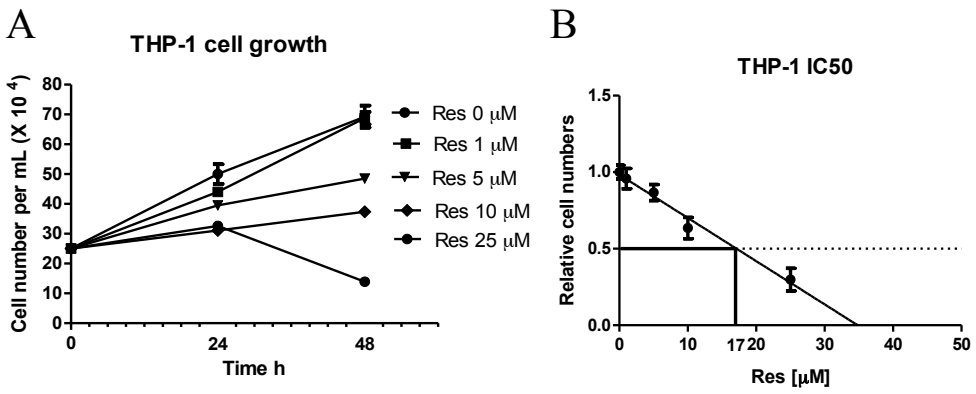


Figure 2. Effect of resveratrol on the cell growth and IC50 in THP-1 monocytes and P-THP-1 macrophages. THP-1 monocytes (1×10^5/mL) were plated in 6-well plates treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. Total cell count of THP-1 cells was measured using Trypan Blue staining after 24 and 48 hours (Fig. 2A). Fig. 2 (B) showed the half maximal inhibitory concentration (IC50) of resveratrol for THP-1 and P-THP-1, respectively. Values are expressed as the mean ± SE of 3 separate experiments.

2.3.2 THP-1 cell cycle analysis and inducement of apoptosis by resveratrol

Cell cycle analysis was performed to examine the influence of Res (1, 5, 10, 25μM) on the progression of THP-1 cells through the cell cycle (Figure 3). Res 1 μM did not have any effect on cell cycle progression, compared to control. Res 5 and 10 μM caused a 1.2- and 2.0-fold increase, respectively, in % cells in S phase, compared to control. This suggests that there was a block or depressed progression through the S phase. In contrast, Res 25 μM increased the % cells in G0/G1 phase by 57.9%, indicating arrest in this phase. A 17-fold increase in sub G0/G1 was seen with res 25 μM, indicating significant apoptosis at this high dosage.

Effect of resveratrol on the cell cycle progression of THP-1 monocytes

A

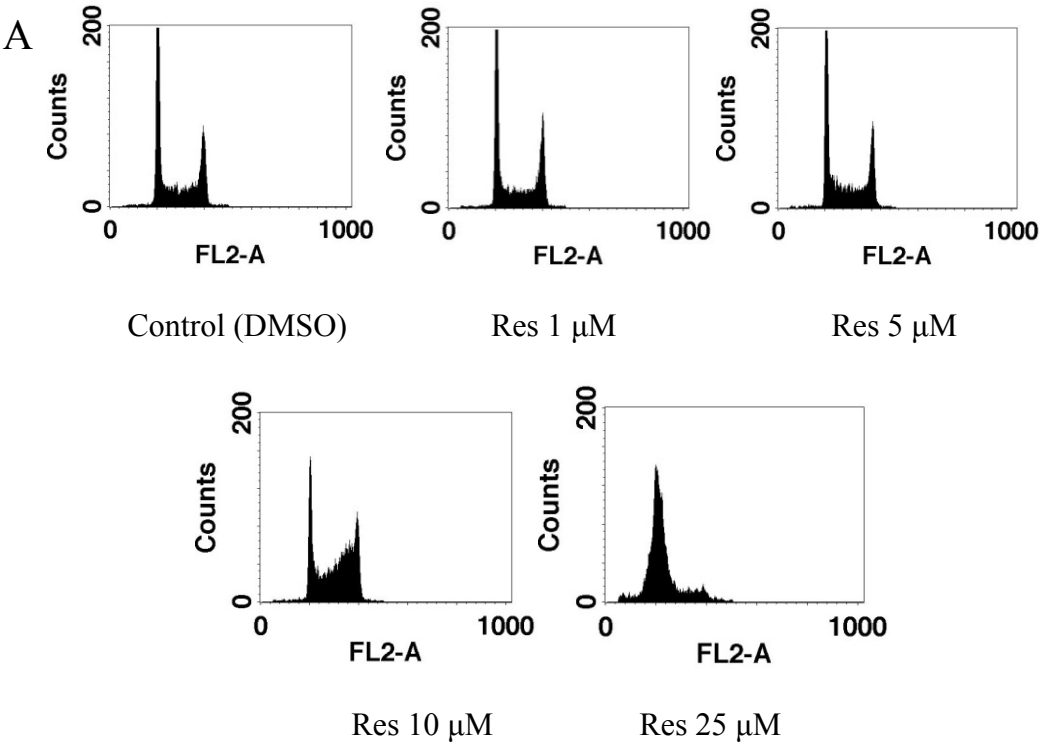


Fig. 3A

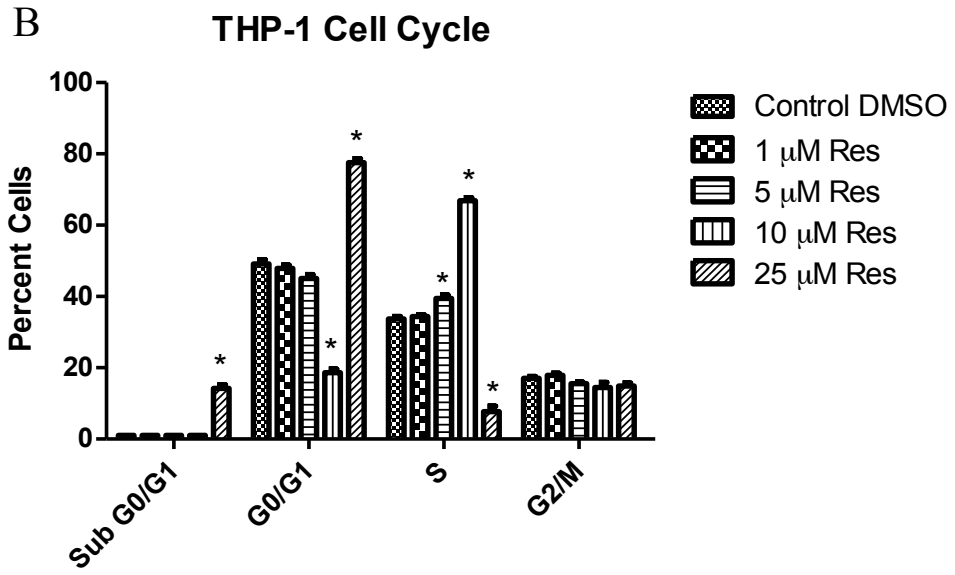


Fig. 3B

Figure 3. Effect of resveratrol on cell cycle progression of THP-1 monocytes. THP-1 monocytes (1×10^5/mL) were plated in 75-cm^2 flasks for 24 hours and then treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. (A) Cell cycle progression was examined using flow cytometry. (B) Cell cycle distribution of collected cells was determined by using flow cytometry. Apo, apoptosis=Sub G0/G1. Percentage of cells in each phase of the cell cycle (sub G0/G1, G0/G1, S, and G2/M) was calculated using CELL Quest. Values were expressed as the mean ± SE of 6 separate experiments. * was used to indicate significantly different from control at each phase of cell cycle ($p<0.01$).

2.3.3 Effect of resveratrol on cell proliferation gene expression in THP-1 monocytes

p21 and p27 are genes related to cellular proliferation. The mRNA level of these genes was determined to examine the mechanism of Res inhibition of expression of these genes in THP-1 cells. Cell cycle analysis indicated that high concentration of Res at 25 μM induced apoptosis in THP-1 cells. Res, starting at 5 μM, also inhibited growth of THP-1 cells.

Increases of 7.2-fold and 25.8-fold in p21 mRNA expression were found in THP-1 cells treated with Res 10 and 25 μM, respectively (Figure 3A left). The expression of p21 protein (Figure 3B) was increased as Res treatment dose increased. Increased expression was first detected with Res 5 μM (3.1-fold). This expression was increased to 3.6- and 4.1-fold, respectively, using Res 10 and 25 μM. Res had no effect on p27 mRNA expression in THP-1 cells (Figure 3A right). Together, these findings suggest Res may induce a p27-independent/ p21-dependent apoptotic pathway in THP-1 monocytes.

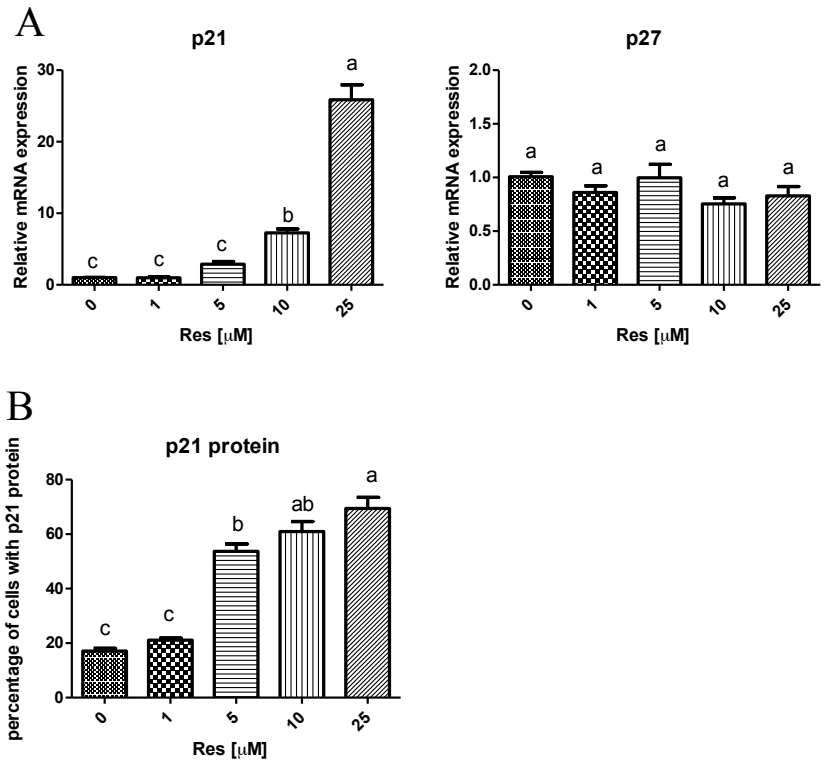


Figure 4. Regulation of cell proliferation related gene expression in THP-1 monocytes treated with resveratrol. THP-1 monocytes were grown for 24 hours and then treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. p21 and p27 mRNA expression (A) was measured by using quantitative RT-PCR. Values were expressed as mean ± SE of 3 separate experiments. Percentage of cells expressing p21 protein (B) was measured by using flow cytometry, according to manufacturer's recommendations. Means with different letters were significantly different ($p<0.05$). p21: cyclin-dependent kinase inhibitor 1A (CDKN1A). p27: cyclin-dependent kinase inhibitor 1B (CDKN1B).

2.2.4 Effect of resveratrol on pro-inflammatory cytokine expression in THP-1 cells after LPS or PAM stimulation

Exposure of THP-1 cells to a dosage of Res (1, 5, 10 μM), attainable by dietary intake, may have a different effect on cellular marker expression than a pharmaceutical dose (25μM). The expression of pro-inflammatory cytokines was determined to address this question. Cells were treated with the inflammatory stimulants LPS and PAM. DMSO treatment served as a control. THP-1 cells had increased marker expression only at the pharmaceutical dose of 25 μM resveratrol (Figure 4C).

IL-1β- IL-6, IL-8, MCP-1, COX-2 and TNF-α mRNA expressions were all enhanced significantly compared to control after LPS and PAM stimulation (Figures 4A and 6B). No change in expression was seen in LPS stimulated cells with Res 1 μM. A dose dependent increase in expression of IL-1β expression was seen with Res 5 to 25 μM. A similar increase in IL-6 was seen with Res 10 and 25 μM. No increase in MCP-1 or COX-2 expression was seen until a dose of Res 25 μM was reached.

No change in THP-1 expression was observed using dietary doses of Res and stimulation with PAM. Increased mRNA expression of IL-1β (2.3-fold), IL-6 (3.8-fold), IL-8 (4.8-fold), and TNF-α (2.0-fold) was seen in THP-1 cells treated with Res 25 μM and PAM.

Effect of resveratrol on pro-inflammatory cytokine mRNA expression in THP-1 monocytes after stimulation with LPS

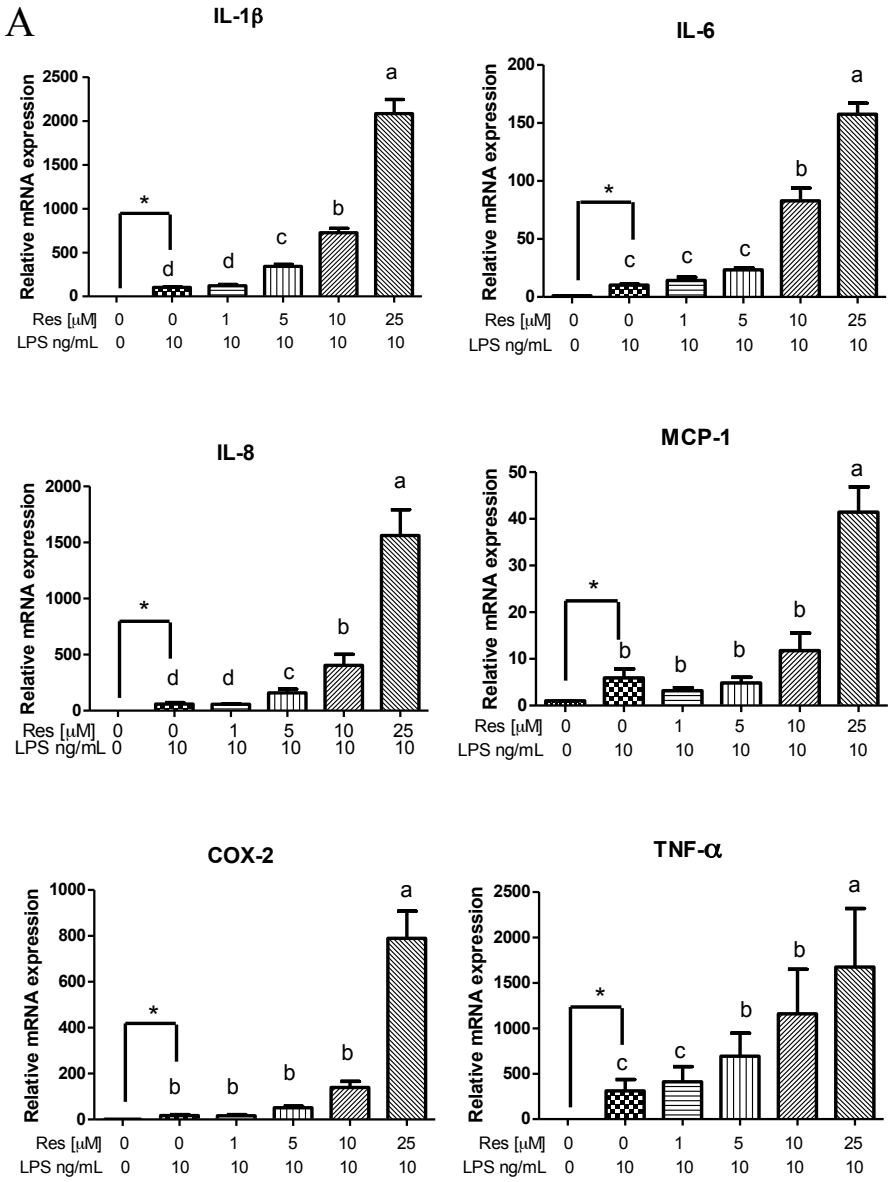


Fig. 6A

Effect of resveratrol on pro-inflammatory cytokine mRNA expression in THP-1 monocytes after stimulation with PAM

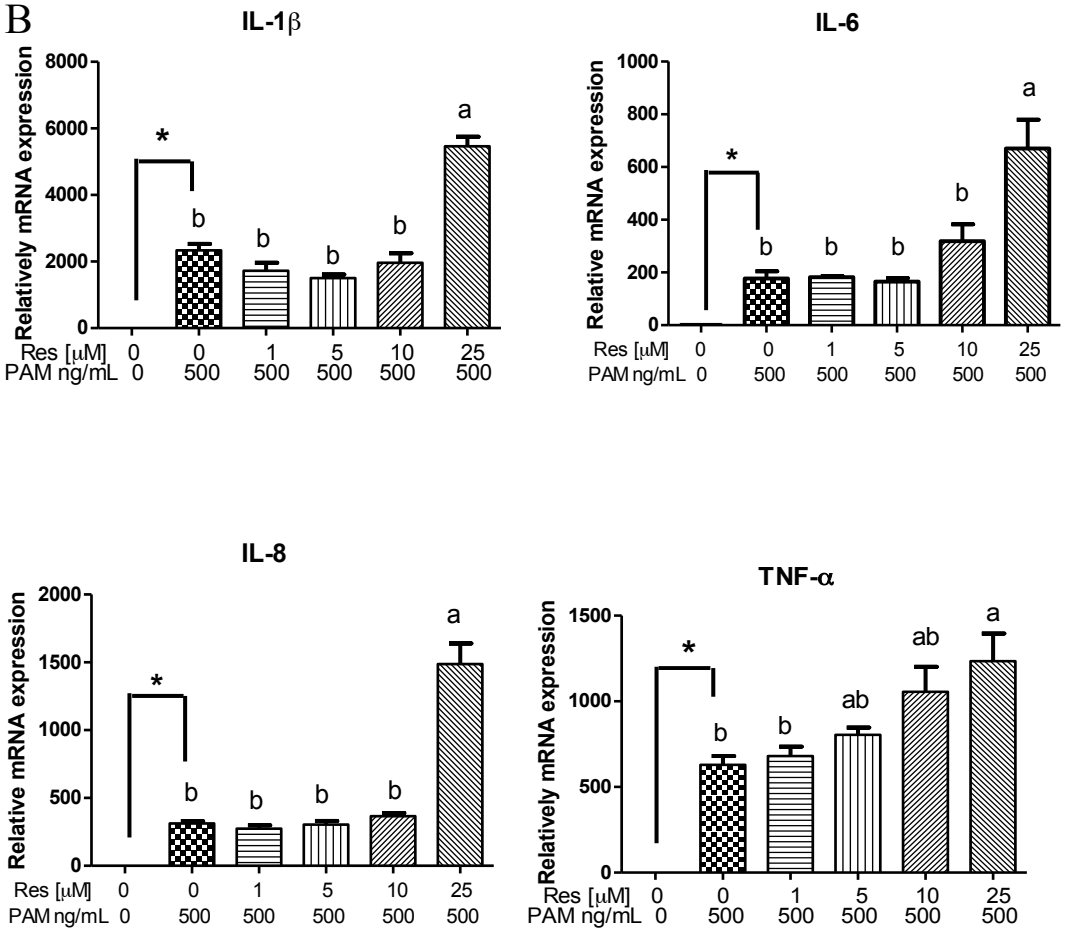


Fig. 6B

Effect of resveratrol on pro-inflammatory cytokine mRNA expression in THP-1 monocytes without stimulation

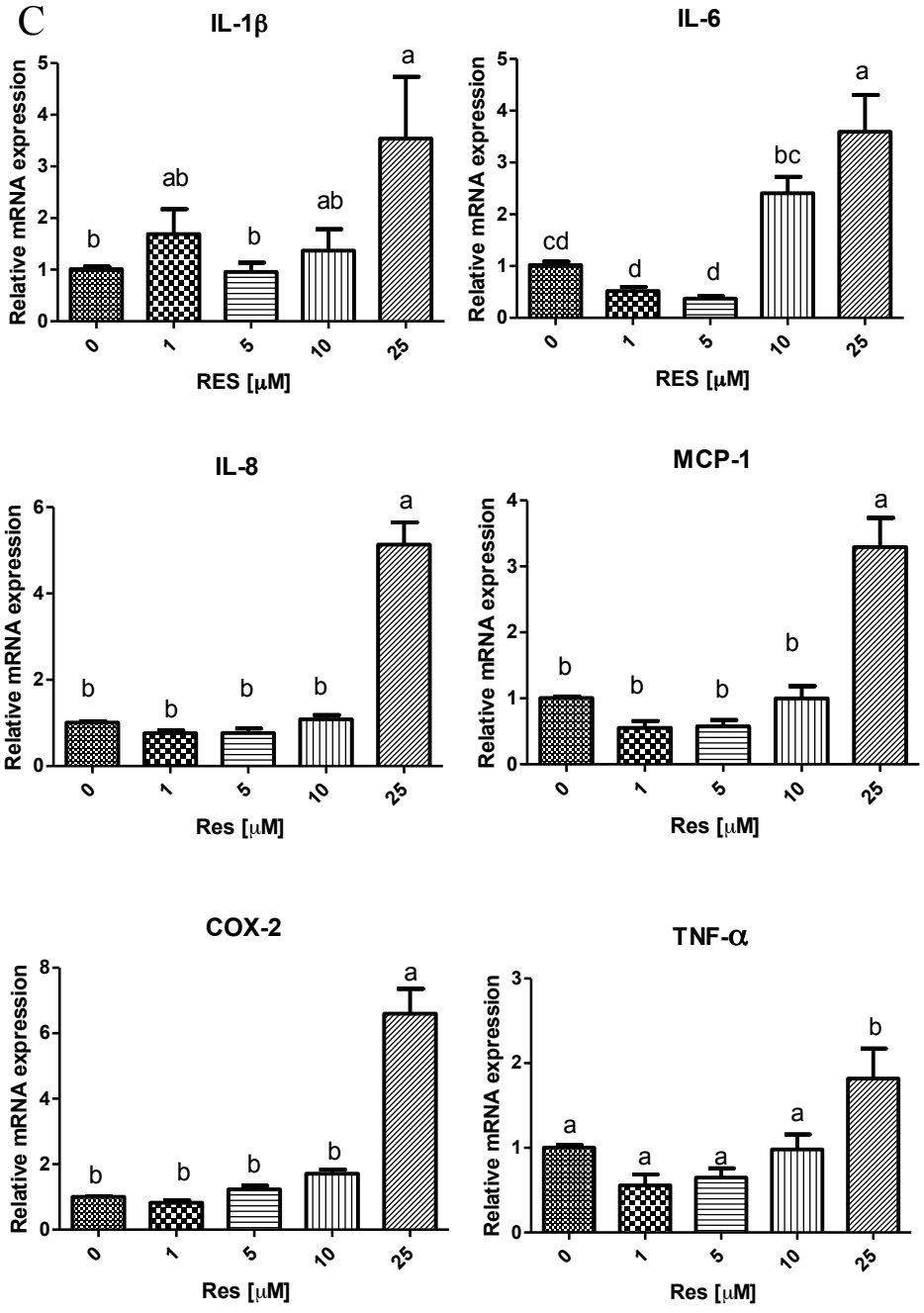


Fig. 6C

Figure 5. Effect of resveratrol on pro-inflammatory cytokine expression in THP-1 monocytes after LPS or PAM stimulation. THP-1 monocytes (2.5×10^5/mL) were cultured for 24 hours and then treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. (A) 10 ng/mL LPS were added to each well and incubated at 37 °C for 2 hours. (B) 500 ng/mL PAM were added to each well and incubated at 37 °C for 2 hours. (C) Cells were incubated at 37 °C for an additional 2 hours only with resveratrol and DMSO treatment. Cytokine mRNA expression was measured by using quantitative RT-PCR. Values were presented as mean ± SE of 3 separate experiments. Means with different letters were significantly different ($p<0.05$). * was used to indicate means are significantly different from control ($p<0.01$).

2.4 Discussion:

Resveratrol is currently sold in the market as supplements and advertised to exert anti-cancer, anti-oxidant and anti-inflammation functions, which were observed in numerous *in vitro* studies. However, the effect of Res at concentrations attainable by dietary intake is lacking from published in vitro studies. Thus, this study was designed to establish how Res treatment duration and concentrations, attainable by dietary intake, might impact on its anti-inflammation function. THP-1 monocytes, derived from a leukemia cell line, were chosen to use in our study, because not only for the economic reason, but also mainly because the effects on both cancer and inflammation can be studied in the present research.

In our study, the influence of Res on THP-1 cell proliferation, cell cycle and apoptosis, pro-inflammatory cytokines and mediators' gene expression was examined to determine its effect related to cancer prevention and chronic inflammation. In **chapter 3**, the specificity of Res on cell type and on its stilbene structure will be further discussed.

Firstly, we examined the influence of Res concentration, ranging from dosages attainable by diet to therapeutic level, on THP-1cells. The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. Our results showed that IC50 for THP-1 monocytes treated with Res was 17 μM, indicating that Res at 17 μM caused half of the cells death. Res is considered to be toxic for human monocytes because of its low concentration to reach IC50.

From cell cycle results on THP-1 cells, Res induced anti-proliferation effect on cell growth with different cell cycle phase arrest in a dose-dependent manner. The effects

of Res on cell cycle are clearly variable depending on the concentration of Res. At lower dosages, Res (5 and 10 μM) just induced S-phase arrest for inhibiting the proliferation of THP-1 without apoptosis. However, Res at 25 μM appeared to be toxic, and induced G0/G1 phase arrest and apoptosis in THP-1 monocytes.

Other indicators, p21 and p27, also showed partial and/or alternate mechanism on how Res regulates cell proliferation in THP-1 monocytes. p21 / WAF1 is also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1, which in humans is encoded by the CDKN1A gene. The p21 protein binds to and inhibits the activity of cyclin-CDK2 or -CDK1 complexes, and thus functions as the p53-dependent (tumor suppressor gene) regulator of cell cycle progression at G1. On the other hand, the p21 protein can also interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair. In our study, Res gradually increased p21 mRNA (starting from 10 μM) and protein expression (starting from 5 μM), whereas p27 mRNA expression was not influenced by Res treatment. Makoto Akashi *et al*., [68] reported that THP-1 is p53-deficient leukemia cell line. The transcription of p21 is directly regulated by p53 [69], and if p53 is mutated, such cells cannot induce p21 expression due to p53 mutation. However, in 1994, Michieli *et al*., demonstrated that p21 is capable of inducing a p53-independent pathway [69]. Thus, the induction of p21 by Res in THP-1 cells, together with cell cycle data, supported that the Res induced p53-independent p21 gene expression may be responsible for blocking cell-proliferation and inducing S phase arrest (Res 5 and 10 μM) or G0/G1 arrest (Res 25 μM) depending on Res concentration. Res is also suggested to be a potential candidate for the treatment of p53-mutant and multidrug-

resistant leukemia cells, but not the main chemotherapeutic agent due to its low IC50 on certain cells.

For the effect of Res on pro-inflammatory cytokines and mediators in THP-1 monocytes, we will further discuss later in **chapter 3 discussions** together with results from P-THP-1 macrophages.

2.5 Conclusion:

Though resveratrol has been studied for more than two decades, its effects on innate immune cells under physiologic attainable conditions are seldom examined. In our study, the first aim is to determine the effect of Res in THP-1 monocytes regarding the toxicity and cancer prevention function. Results from the present study showed that Res exerted anti-proliferation on THP-1 cells, induced apoptosis on THP-1 cells with pharmaceutical concentration, as well as anti-cancer function by elevating p21 gene expression, together with S-phase arrest and G0/G1-arrest in cell cycle with dietary and pharmaceutical concentrations, respectively. Our conclusion is that resveratrol exhibited concentration-dependent toxicity, induced different cell cycle arrest in a concentration manner, and exhibited blocking of cancer development through the induction of p53-independent p21 gene expression in human THP-1 monocytes.

Chapter 3 Res exerts cell type or inherent stilbene structure specificity in the regulation of inflammation mediators

3.1 Introduction:

3.1.1 Monocytes and macrophages

Monocytes and macrophages are part of the immune system. Monocytes circulate through the bloodstream for one to three days, and then travel into specific tissues to differentiate into resident macrophages or dendritic cells (specifically to replenish macrophages or dendritic cells) by receiving the inflammation signals [13]. Monocytes can move quickly (8-12 hours) to sites of infection in the tissues and differentiate into macrophages and dendritic cells to elicit an immune response.

Both macrophages and monocytes function in innate immunity defense. Besides protecting our body as first barrier from non-specific infection, macrophages can also help to initiate specific defense mechanisms (adaptive immunity) in mammals [70]. Several cell types (macrophages, neutrophils, dendritic cells and B cells) in the immune system engulf microorganisms via phagocytosis. To aid in the destruction of microorganisms, several important factors are release within phagolysosome: phagocyte oxidase, nitric oxide, anti-microbial proteins, anti-microbial peptides, binding proteins and hydrogen ion transport [70].

3.1.2 Chronic inflammation and cancer:

Inflammation is a sword with two edges. Acute inflammation is the defense of infections, however, on the other side, chronic inflammation showed a link with one fourth of cancer incidence by failing in the precise immune regulations and cellular homeostasis. Previous research established that the mediators from the inflammation response could cause both genetic and epigenetic changes (mutations on p53, DNA methylation and post-translational modifications) and lead to uncontrolled development and progression of cancer. Through inflammation, mediators are secreted to induce numerous cascade-signaling pathways, including reactive oxygen and nitrogen species, cytokines, prostaglandins and growth factors. Substantial evidence [71, 72] indicated that inflammation mediators can promote cancer development.

In activated inflammatory cells, the enhanced production of reactive oxygen and nitrogen species (e.g., OH•, NO•, O_2•, OONO•) can further induce lipid peroxidation, reactive aldehydes-malondialdehydes (MDA) and 4-hydroxynonenal (4-HNE). Both MDA and 4-HNE are potential cancer inducers capable of point-mutating tumor suppressor genes [73]. Interestingly, NO• can both activate p53 tumor suppressor pathway and induce oncogenic mutations on p53 gene in a concentration-dependent manner [74]. With low concentration, NO• can induce p53 post-translational modification through ATM and ATR signaling pathways to accumulate p53, which can inhibit cell growth and induce apoptosis. However, mutated p53 accumulates as a result of this action. On the other hand, a higher amount of NO• helped to develop lymphoma and sarcomas in p53-deficient mice. NOS2 (nitric oxide synthase 2) expressed high amount of NO• for a longer time whereas NOS1 and NOS3 just produce low level of

NO• [75]. Mutation of p53 was found to increase in those patients who had colon inflammation [76].

There are two kinds of cytokines: pro- inflammatory (maintain- inflammation and anti-inflammatory (reduce inflammation and promote healing) ones, which can further induce or inhibit tumor development [77]. Under normal situation, cytokines are initiated and terminated under real attack (e.g., bacteria, virus, infection, trauma). However, uncontrolled and sustained production of cytokines can lead to chronic inflammation rather than acute inflammation, and alter cell functions and cell type. Pro-inflammatory cytokines include IL-1, IL-6, IL-8, TNF-α, CSF and MIF (table 1), which increase vascular adhesion molecules, vascular endothelial growth factor-VEGF and other growth factors and then contribute to angiogenesis and metastasis in cancer progression [78]. Carcinogens are found in foods, cigars, and industrial pollutions, which are reported to increase most of the pro-inflammatory cytokines and chemokines [79]. One good example is chronic infection of *Helicobacter pylori* and gastric carcinoma. *Helicobacter pylori* (*H. pylori*) are micro-aerophilic spiral-shaped Gram-negative bacteria, which in the stomach. *H. pylori* infection affects 50% of the population all over the world [80]. Specially, there are much more incidence of stomach cancer in some part of Asia [81]. One Korean study on Asian population also reported an increased risk of gastric cancer, which was associated with an elevated IL-1β expression in *H. pylori*-infected patients [82]. Furthermore, many reports showed that elevated expression of IL-8, IL-6, CSF, MIF and TNF-α are associated with tumor development [83, 84, 85]. IL-8, originally named as monocyte-derived neutrophil chemotactic factor (MNDCF), can act as autocrine growth factor to help angiogenic activities in several cancer types [86, 87]. IL-6

can induce anti-apoptotic genes Bcl2 and Bcl-XL to suppress anti-cancer function by mediating pRb hyperposphorylation [88, 89]. MIF has a strong association with cancer by regulating p53 suppressor genes, cell cycle and senescence. In addition, MIF is also involved in increasing COX-2 and NOS2, inhibiting apoptosis and activating NF- κB [90]. NF-κB is an important transcription factor that induce cancer during chronic inflammation, whose role is to inhibit apoptosis and rather than to elevate cell progression, angiogenesis and metastasis [91,92]. Furthermore, COX-2, stimulated only by inflammation, hypoxia and Wnt-signaling [93], has been reported to promote cancer in epithelial cell and stroma by elevating Bcl2 expression and members of matrix metalloproteinase family [94].

3.1.3 Inflammation and vascular disease:

Lots of evidence from cohort studies support the correlation between chronic inflammation and cardiovascular risk, that the inflammatory parameters (fibrinogen, C reactive protein, and serum amyloid A), as well as cellular adhesion molecules and cytokines, are all associated with increased risk of vascular and heart disease. From Dr. P .M. Ridker's review [95], he mainly reviewed the relationship between C reactive protein (CRP) and epidemiology of coronary risk. In response to acute inflammation, infection and stimuli, the liver secrets IL-6 and elevates CRP rapid responses in the blood. However, with the loss of homeostasis of pro-inflammatory cytokines, CRP may lead directly to chronic inflammation and future coronary events. High-sensitivity assays for CRP (hs-CRP) are used to evaluation the extent of chronic vascular inflammation. An 8-year cohort study, which involved 22,000 healthy men, found that those in the highest quartile of hs-CRP compared to those with lower level of hs-CRP, have two-fold

elevation in stroke, three-fold increase in the risk of myocardial infarction, and a four-fold enhancement in developing peripheral vascular disease. Further, similar results were also reported in elder people and women [96, 97].

On the other hand, available evidence indicated that active cytokines secretion resulted in endothelial cell injury and initiation of arterial-wall inflammatory processes. There is another link between periodontal disease and inflammation. Periodontal disease is initiated by bacterial attack orally around the gum, which leads to release of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α). Thus, such action shares an association between periodontal disease and coronary disease [98].

3.1.4 Anti-inflammation effect of Res

Recently, studies [99] revealed that resveratrol exerted anti-inflammation function by inducing antioxidant activity, blocking NF-κB signaling pathway and other pro-inflammatory mediators (figure 6). Firstly, Res exerts anti-oxidant property by reducing hydrogen peroxide-induced cytotoxicity and intracellular accumulation of ROS [100, 101, 102]. In chronic inflammation, macrophages remain activated and continue to release pro-inflammatory signals to induce further trauma, DNA mutant and other chronic disease including cardiovascular disease, type II diabetes, brain damage and cancer. TNF-α is a potential target for triggering other pro-inflammatory cytokines (IL-1, IL-6, IL-8, granulocyte macrophage-colony stimulating factor-GM-CSF). In 2009, Res was found to inhibit LPS-induced TNF-α, IL-1β, IL-6 expression in mouse monocytes [103]. In angiotensin II induced adhesion of leukocytes, Res reduced cellular adhesion molecule expression, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1-alpha (MIP-1α) [104]. Kang *et al.* and Olholm *et al.* presented further

confirmation of inhibition on such cytokines, TNF-α and IL-6, that both were decreased in either LPS-induced RAW264.7 mouse macrophages by Res (0.1-10 μM) [110] or in human adipose tissue explants [105].

COX-2, named cyclooxygenase-2 or prostaglandin-endoperoxide synthase2 (PTGS2), is an enzyme that induces prostanoids expression and inflammation. Physiological symptoms are fever and pain. Researchers found that increased COX-2 was associated with inflammation and certain cancers [106, 107, 108]. In 1998, Subbaramaiah *et al.,* demonstrated that Res inhibited prostaglandin E2-PGE2 by inhibiting COX-2 expression [109]. Recently, Kang *et al*., (2009) reported that resveratrol significantly inhibited the PMA plus A23187-induced TNF-α, IL-6 and IL-8, as well as COX-2 expression in mast cells (HMC-1) [110].

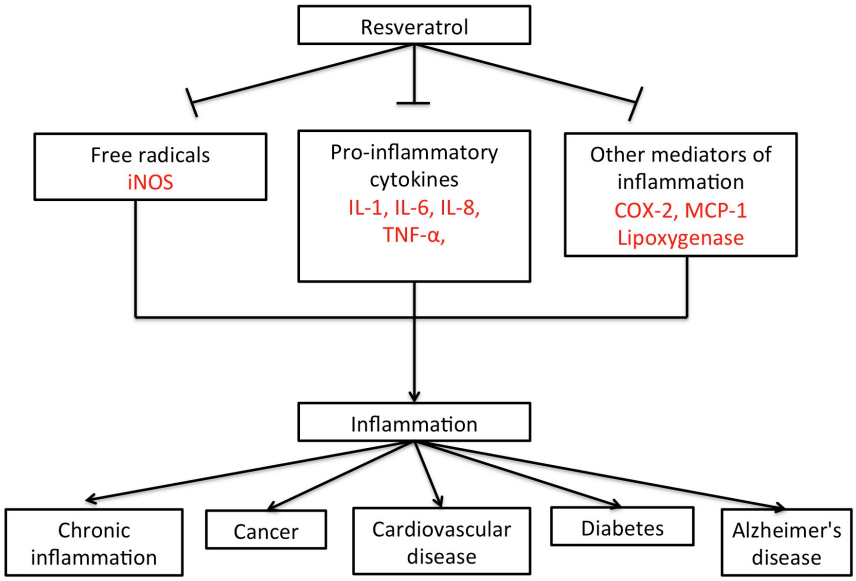


Figure 6. Anti-inflammatory function of Resveratrol in several different signal pathways. iNOS (inducible NO synthesis), IL (interleukin), TNF-α (tumor necrosis factor-α), MCP-1 (monocyte chemotactic protein-1), Chemokine ligand 2 (CCL-2), COX-2 (Cyclooxygenase 2).

3.1.5 Hypothesis:

In chapter 2, we have tested the effect of Res in THP-1 monocytes related cell viability and cancer prevention by induced p21 elevation and cell cycle arrest. In chapter 3, we further examined the specificity of Res on either cell type or its stilbene structure. In our study, we compared the cell viability from THP-1 monocytes and P-THP-1 macrophages, and the pro-inflammatory cytokines productions in both cell lines induced by LPS or PAM inflammation. Furthermore, to figure out why Res has such effects at the molecular level, we compared it with two other bioactive compounds, one shared the same stilbene structure, and another one possessed no stilbene structure, in both THP-1 and P-THP-1 cells treated with LPS/PAM inflammation inductions.

3.2 Materials and methods:

3.2.1 Materials and reagents:

Cell culture media, DMEM (Cat #11,995) and RPMI 1,640 (Cat #11,875), were purchased from GIBCO (Grand Island, NY, USA). Lipopolysaccharides (derived from *Escherichia coli* 0111:B4), resveratrol (Res), pterolstilbene (Ptes), genistein (Gen) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Strata Script First Strand Complementary DNA Synthesis kit was obtained from Stratagene (Santa Clara, CA, USA). TaqMan Universal PCR Master Mix for Real-time PCR was purchased from Applied Biosystems (Carlsbad, CA, USA). More material information can be found in table 2.

3.2.2 Cells and cell growth conditions:

THP-1 cells:

Human THP-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1,640 media, which contained 10% fetal bovine serum (FBS) and 1% amphotericin B/streptomycin/penicillin, hereinafter referred as the complete medium (CM), and were incubated at 37 °C in humidified ambient air with 5% CO_2. Prior to experiments, THP-1 cells were grown in the medium for 24 hours for adaption. After 24 h, the medium was removed and replaced by fresh CM with different concentrations of treatments (resveratrol, pterostilbene, and genistein).

PMA-differentiated THP-1 macrophages (P-THP-1):

THP-1 monocytes (5×10^5 cells/mL, 2mL) were seeded in CM in 6-well plate (Costar; Corning Incorporated, Corning, NY, USA). Cells were then differentiated with PMA (25ng/mL) for 48 hours, which were incubated at 37 °C with 5% CO_2 in ambient air. After washing three times with PBS, attached cells were grown in fresh CM and ready for treatments.

3.2.3 Total protein analysis on P-THP-1 macrophages

Total protein of P-THP-1 was measured to determine cell growth/viability. After P-THP-1 cells (5×10^5 cells/mL, 2mL) were differentiated with 25 ng/mL PMA in 6-well plates for 48 hours under cover of foil, which were incubated at 37 °C with 5% CO_2 in ambient air. P-THP-1 cells were then treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. Cells were harvested and total protein was measured using the sulforhodamine B (SRB) assay. Cells were first fixed with trichloroacetic acid (TCA, 10%) for one hour at 4 °C, and then stained for 20 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by five washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base (pH 10.5) for determination of optical density in a computer-interfaced, 96-well microliter plate reader (530 nm) [111, 112].

3.2.4 Total RNA isolation, cDNA synthesis, and gene expression analysis using real-time PCR

The mRNA expression level on THP-1 and P-THP-1 cells was measured by real-time PCR. THP-1 and P-THP-1 cells were cultured in 6-well plates treated with control

(DMSO), resveratrol (1, 5, 10, 25 μM), pterostilbene (Ptes) (10 μM), Genistein (Gen) (10 μM) for 48 hours and then LPS (10 ng/mL) or Pam3CSK4 (500 ng/mL) were added for another 2 hours. Methods of gene expression analysis by RT-PCR are presented in **chapter 2.2.5**. The TaqMan gene expression assay was used to measure IL-1β, IL-6, IL-8, COX-2, MCP-1, TNF-α, CYP1A1 and CYP1B1. The mRNA levels were normalized to an internal control, GAPDH mRNA.

3.2.5 IL-6 protein measurement by ELISA

To determine IL-6 protein expression, THP-1 monocytes (5×10^5 cells/mL) were cultivated in 14-mL EP tubes overnight, and treated with DMSO, Res (1, 5, 10, 25 μM), Ptes (10 μM), or Gen (10 μM) for 48 hours. Medium and treatments (Res, Ptes, Gen) were changed every 24 hours. LPS (10 ng/mL) or Pam3CSK4 (PAM) (500 ng/mL) were added with previous treatment for another 4 hours. Then supernatant was collected for ELISA test while remaining cells were counted for normalization of quantity.

After 48-hour of differentiation by using PMA (50 ng/mL) to obtain the P-THP-1 macrophages (1×10^6 cells/mL), Res (1, 5, 10, 25 μM), Ptes (10 μM) and Gen (10 μM) were added for 48 hours. Medium and treatments were changed every 24 hours. After 48 hours, LPS (10 ng/mL) or PAM (500 ng/mL) were added with Res, Ptes and Gen for 6 hours. Cells were collected for ELISA test, and results were normalized by total protein content (SRB assay) of P-THP-1 cells.

3.2.6 Statistics:

Data were analyzed with GraphPad Prism 5 and are presented as means ± SE. A one-way ANOVA followed by post hoc analysis using the Tukey test to determine the statistical significance among treatments, $p < 0.05$.

3.3 Results:

3.3.1 Effect of resveratrol on the growth of P-THP-1 macrophages

After 48 hours of incubation, Res demonstrated a dose dependent effect on the growth of P-THP-1 cells. Res 1 and 10 μM treated cells had a similar growth rate as the DMSO controls at 48 hours (b, Figure 7A). Res 5 and 25 μM were associated with a 13.2% increment and 14.3% reduction, respectively, in the growth of P-THP-1 cells at 48 hours compared to DMSO controls ($p < 0.05$) (Res 5 and 25 μM, Figure 7A). The IC50 of Res for P-THP-1 cells was 76 μM (Figure 7B).

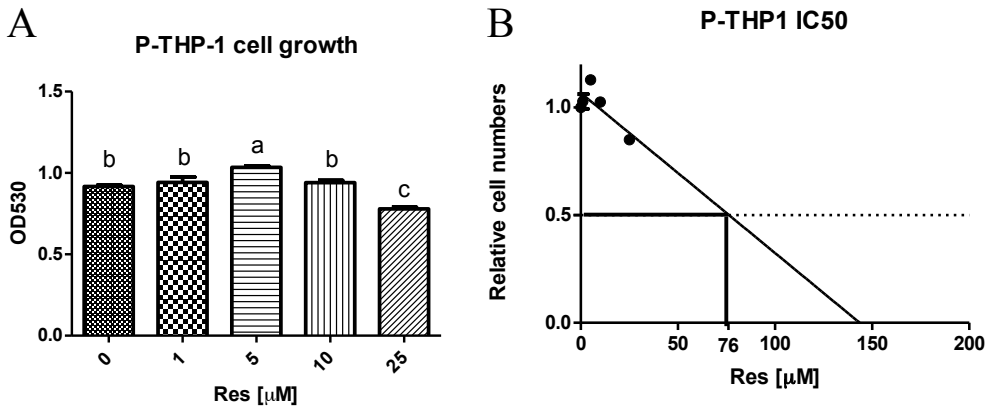


Figure 7. Effect of resveratrol on the cell growth and IC50 in P-THP-1 macrophages. P-THP-1 macrophages (5×105/mL) (A) were plated in 6-well plates treated with (1, 5, 10, and 25 μM) or without resveratrol (control, DMSO) for 48 hours. Total protein content of P-THP-1 cells was measured by using the SRB method and was used to normalize the number of P-THP-1 cells after 48 hours. Fig. 7 (B) showed the half maximal inhibitory concentration (IC50) of resveratrol for P-THP-1. Values are expressed as the mean ± SE of 3 separate experiments. For Fig. 7 (A), means with different letters are significantly different ($p<0.05$).

3.3.2 Differentiation of THP-1 monocytes into macrophages by PMA

Macrophages are considered to be a good cell model for testing *in vitro* anti-inflammatory response. THP-1 monocytes can be differentiated into macrophages after exposure to PMA. Dr. E. K. Park [113] reported that cellular gene expression was not altered after exposure to low concentrations of PMA. THP-1 monocytes were incubated with 10, 25, and 50 ng/mL PMA for 48 hours. Flasks were covered with foil to prevent exposure to light. THP-1 monocyte differentiation into macrophage was documented by using two methods, cell adhesion and morphology as well as expression of the macrophage surface marker CD 14. THP-1 monocytes were round small spherical cells that grow in suspension. THP-1 differentiated macrophages were slightly larger and adhere to the culture flask walls. THP-1 monocytes were fully attached to the culture flask after differentiation using PMA 25 and 50 ng/mL. Adherent cells were examined for morphologic changes and CD 14 mRNA expression. There was a 30-fold increase in CD-14 expression in THP-1 cells treated with PMA (25 ng/mL) (Figure 8), compatible with cell differentiation into macrophages. Both morphologic observation and CD 14 expression confirmed the differentiation of THP-1 monocytes into macrophages. PMA concentrations of 25 ng/mL and treatment time of 48 hours were chosen as the optimal differentiation conditions.

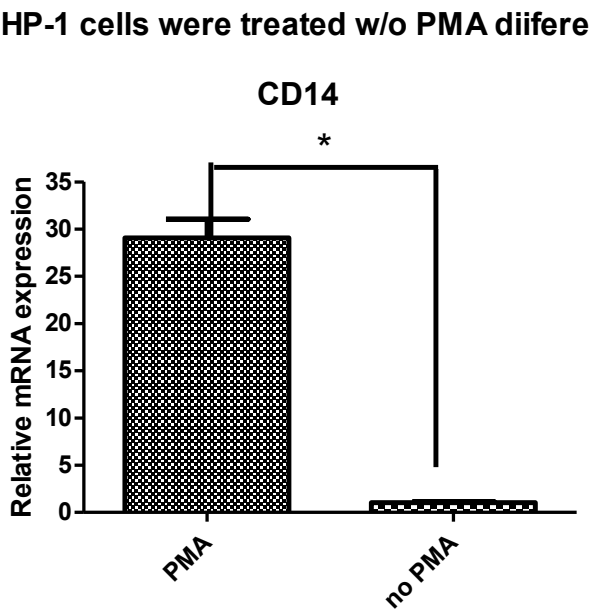
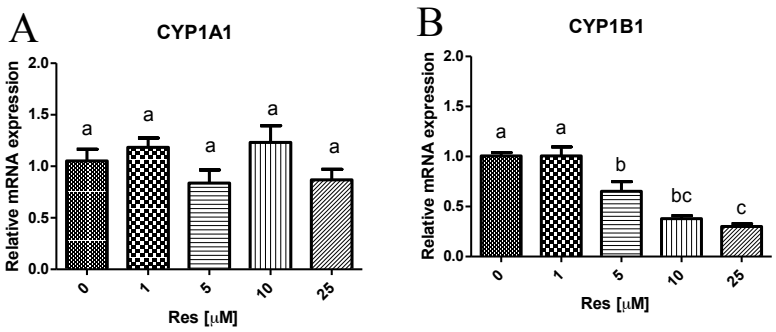


Figure 8. CD 14 is a marker for THP-1 monocyte differentiation into macrophages after PMA stimulation. THP-1 monocytes (5×10^5/ml) were differentiated by using 25 ng/mL PMA for 48 hours in the dark. After stimulation, total RNA was extracted from cell samples, and 1 μg of extracted total RNA was reverse transcribed into cDNA. Quantitative real-time PCR analyses were performed in triplicate. Loading was normalized by using GAPDH as an internal control. THP-1 mRNA expression without PMA stimulation was normalized to a value of 1. Values were expressed as the mean ± SE of 3 separate experiments. * was used to indicate that the means were significantly different ($p<0.01$).

3.3.3 CYPs gene expression

CYP1A1 and CYP1B1 are important genes related to cellular processing of drugs. THP-1 and P- THP-1 cells were examined to determine if Res treatment may influence CYP1A1 and CYP1B1 mRNA expression. CYP1B1 expression was inhibited at doses of Res 5 to 25 μM in both THP-1 (Fig. 9B) and P-THP-1 (Fig. 9C) cells with reductions of 34%-70% and 86%-90%, respectively. In contrast, CYP1A1 mRNA expression was not affected in THP-1 cells (Figure 9A), and was not detected in P-THP-1 cells.

Effect of resveratrol on drug metabolism gene expression in THP-1 monocytes



Effect of resveratrol on drug metabolism gene expression in P-THP-1 macrophages

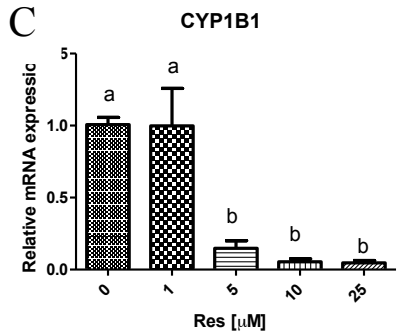


Figure 9. Effect of resveratrol on drug metabolism gene expression in THP-1 monocytes and P-THP-1 macrophages, without LPS stimulation. THP-1 monocytes (A) and P-THP-1 macrophages (B) were treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. CYP1A1 and CYP1B1 mRNA expression was then measured by using quantitative RT-PCR. Values were expressed as the mean ±SE of 3 separate experiments. Means with different letters were significantly different ($p<0.05$).

3.3.4 Effect of resveratrol on pro-inflammatory cytokines expression of P-THP-1 macrophages after stimulation with LPS or PAM stimulation, or without stimulation

LPS stimulation in P-THP-1 with no Res treatment (LPS 10 ng/mL, Res 0 μM) (Fig. 10A) markedly increased mRNA level of IL-1β, IL-6, IL-8, MCP-1, COX-2, and TNF-α, as compared to controls (LPS 0 ng/mL, Res 0 μM). Resveratrol treatment, Res 1-25 μM, exerted no additional increment in IL-1β and IL-8 mRNA levels in LPS stimulated P-THP-1 macrophages. In contrast, Res 5-25 μM treatments markedly reduced IL-6 and COX-2 levels in LPS stimulated cells. In addition, a 51.5% reduction in MCP-1 level was only observed in the Res 25 μM LPS stimulated cells. However, Res 10-25 μM induced 51-55% increment in TNF-α in LPS stimulated P-THP-1 cells.

PAM stimulation in such cells with no Res treatment (PAM 500 ng/mL, Res 0 μM) (Fig. 10B) elevated IL-1β, IL-6, IL-8 and TNF-α mRNA levels as compared to controls (PAM 0 ng/mL, Res 0 μM). In PAM stimulated P-THP-1 macrophages, Res treatment, Res 1-25 μM, mildly depressed IL-1β and IL-8 mRNA levels, but exerted a progressive reduction in IL-6 mRNA levels with increases in Res level. Res treatment exerted no effect at Res 1-5 μM but marked increases at Res 10-25 μM on TNF-α mRNA level in PAM stimulated P-THP-1 macrophages.

In control P-THP-1 macrophages, without stimulations (Fig. 10C), IL-8, COX-2 and TNF-α mRNA expression was depressed around 50%, fairly evenly, from Res 1 to 25 μM treatments. However, IL-1β mRNA level was similarly depressed from Res 5 to 25 μM.

Effect of resveratrol on pro-inflammatory cytokine expression in P-THP-1 macrophages after LPS stimulation

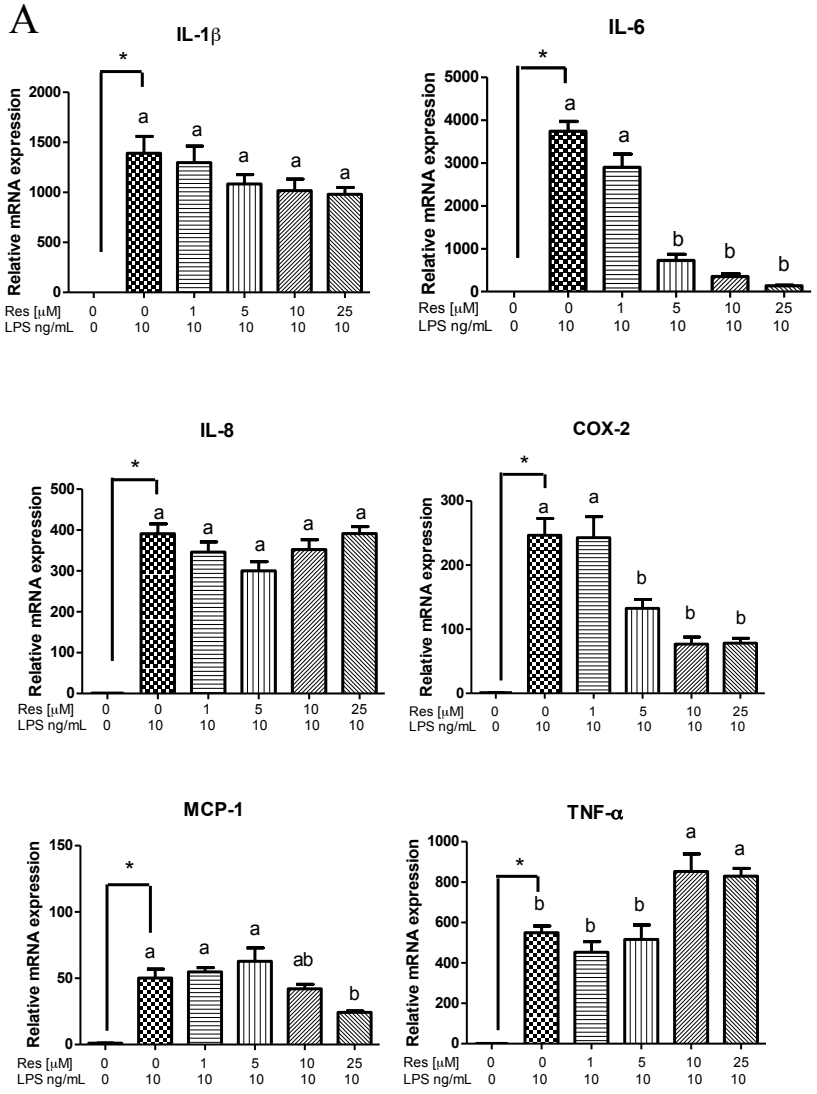


Fig. 10A

Effect of resveratrol on pro-inflammatory cytokine expression in P-THP-1 macrophages after PAM stimulation

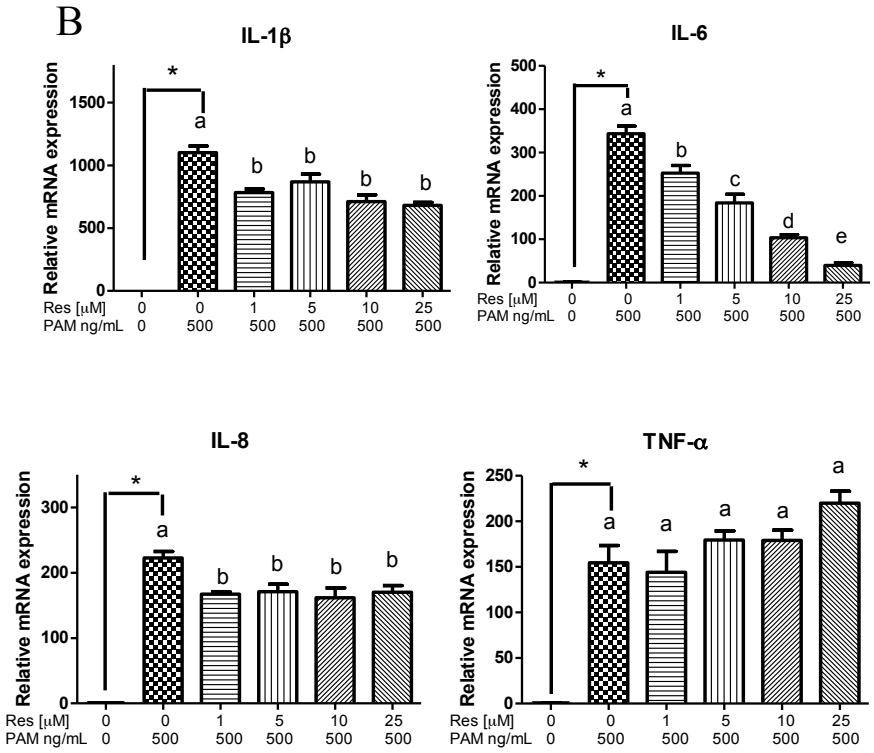


Fig. 10B

Effect of resveratrol on pro-inflammatory cytokine expression in P-THP-1 macrophages without stimulation

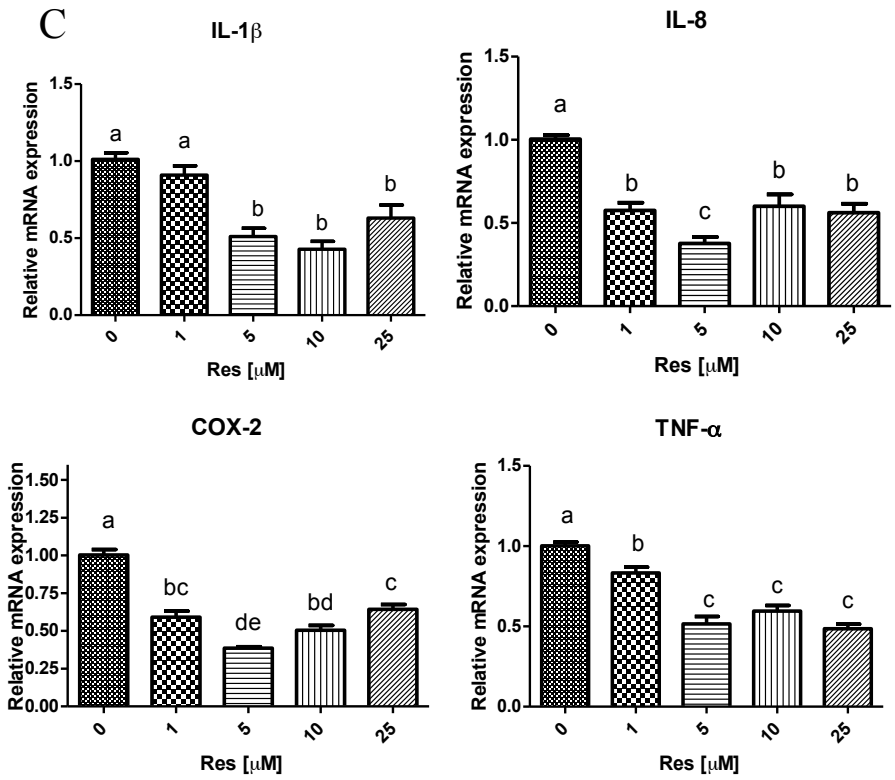


Fig. 10C

Figure 10. Effect of resveratrol on pro-inflammatory cytokine expression of P-THP-1 macrophages with or without LPS or PAM stimulation. THP-1 monocytes (5×10^5/mL) were differentiated by PMA in the dark for 48 hours. P-THP-1 macrophages were then treated with (1, 5, 10, 25 μM) or without resveratrol (control, DMSO) for 48 hours. (A) 10 ng/mL LPS were added to the final cell culture for 2 hours at 37 °C. (B) 500 ng/mL were added to the final cell culture for 2 hours at 37 °C. (C) Control cells treated for 2 additional hours without further treatment. Cytokine mRNA expression was measured by using quantitative RT-PCR. Values were expressed as the mean ±SE of 3 separate experiments. Means with different letters were significantly different ($p<0.05$). * was used to indicate significantly different from control ($p<0.01$).

3.3.5 Comparison of three bioactive compounds

LPS stimulation in cells (Fig. 11A) without bioactive treatment (LPS 10 ng/mL, DMSO 1 μM) markedly decreased IL-1β, IL-6, IL-8, COX-2, MCP-1 and TNF-α mRNA levels as compared to controls (LPS 0 ng/mL, DMSO 1 μM). Resveratrol 10 μM and pterolstilbene (Ptes 10 μM) treatments markedly increased all mRNA expressions with the exception of Ptes, which exerted no effect on TNF-α. Gen 10 μM treatment also had no effect on these mRNA expressions.

In contrast, in THP-1 cells not stimulated by LPS (Fig. 11B), bioactive compounds exerted little or no effect on mRNA expressions. The exceptions are: Res treatment increased IL-1β level by 67.8%; and Gen treatment depressed TNF-α level by 43.7% as compared to DMSO controls.

LPS stimulation in P-THP-1 macrophages (Fig. 11C), without bioactive treatments (LPS 10 ng/mL, DMSO 1 μM), markedly elevated mRNA level of IL-1β, IL-6, IL-8, COX-2, MCP-1 and TNF-α, as compared to controls (LPS 0 ng/mL, DMSO 1 μM). All bioactive compounds exerted no effect on IL-1β level. Most importantly, IL-6, COX-2 and MCP-1 levels were depressed by Res and Ptes, both with inherent stilbene structure. Gen elevated IL-6, but had no effect on COX-2 or MCP-1. Only Ptes treatment, among all bioactive compounds, increased IL-8 and TNF-α mRNA level as compared to control DMSO treatment.

In P-THP-1 macrophages (Fig. 11D), not stimulated by LPS, Res 10 μM treatment depressed mRNA level of IL-1β, IL-8, COX-2, MCP-1 and TNF-α. In addition, Ptes and Gen treatment only decreased mRNA level of IL-1β and COX-2, and MCP-1, respectively, as compared to control (DMSO 1 μM).

Effect of bioactive compounds on pro-inflammatory cytokine expression in THP-1 monocytes after LPS stimulation

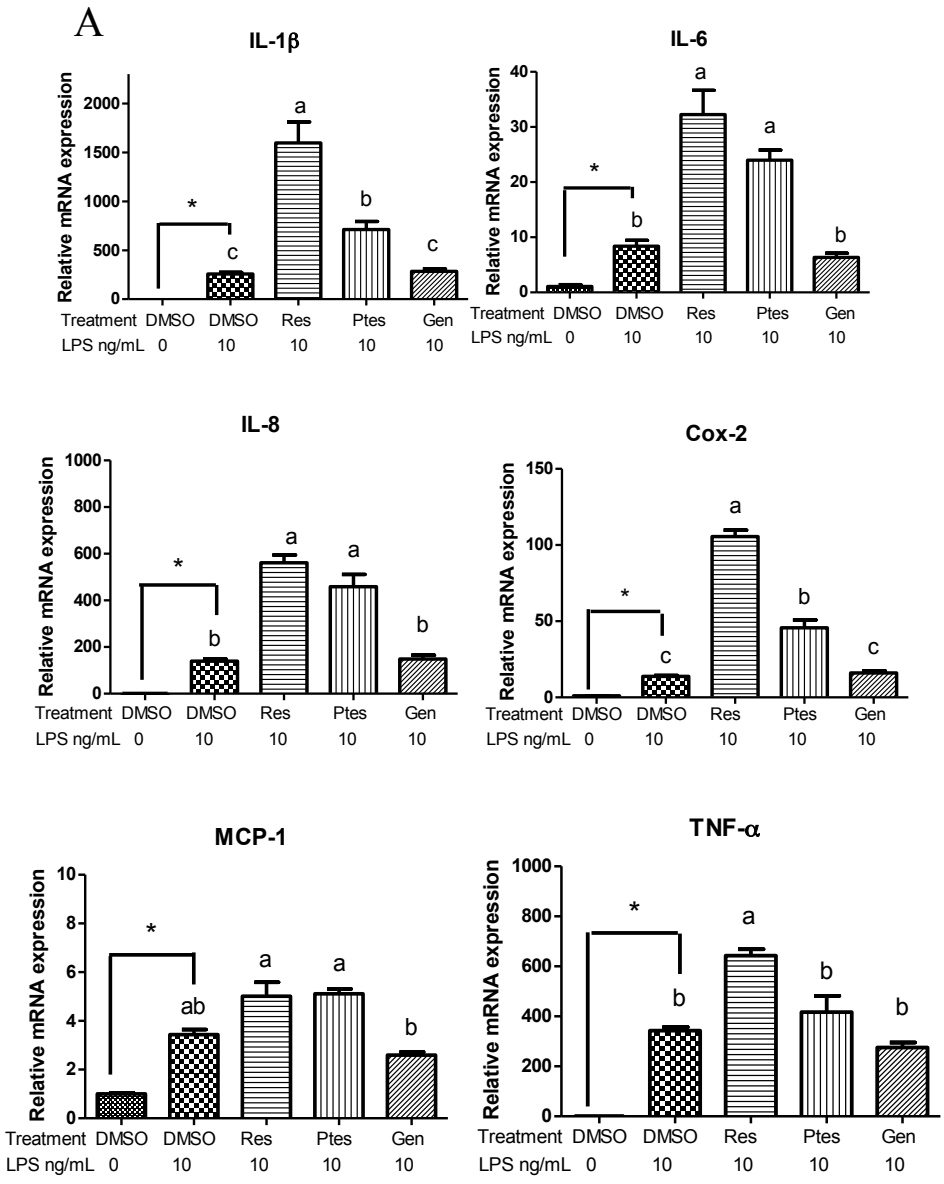


Fig. 11A

Effect of bioactive compounds on pro-inflammatory cytokine expression in THP-1 monocytes with no stimulation

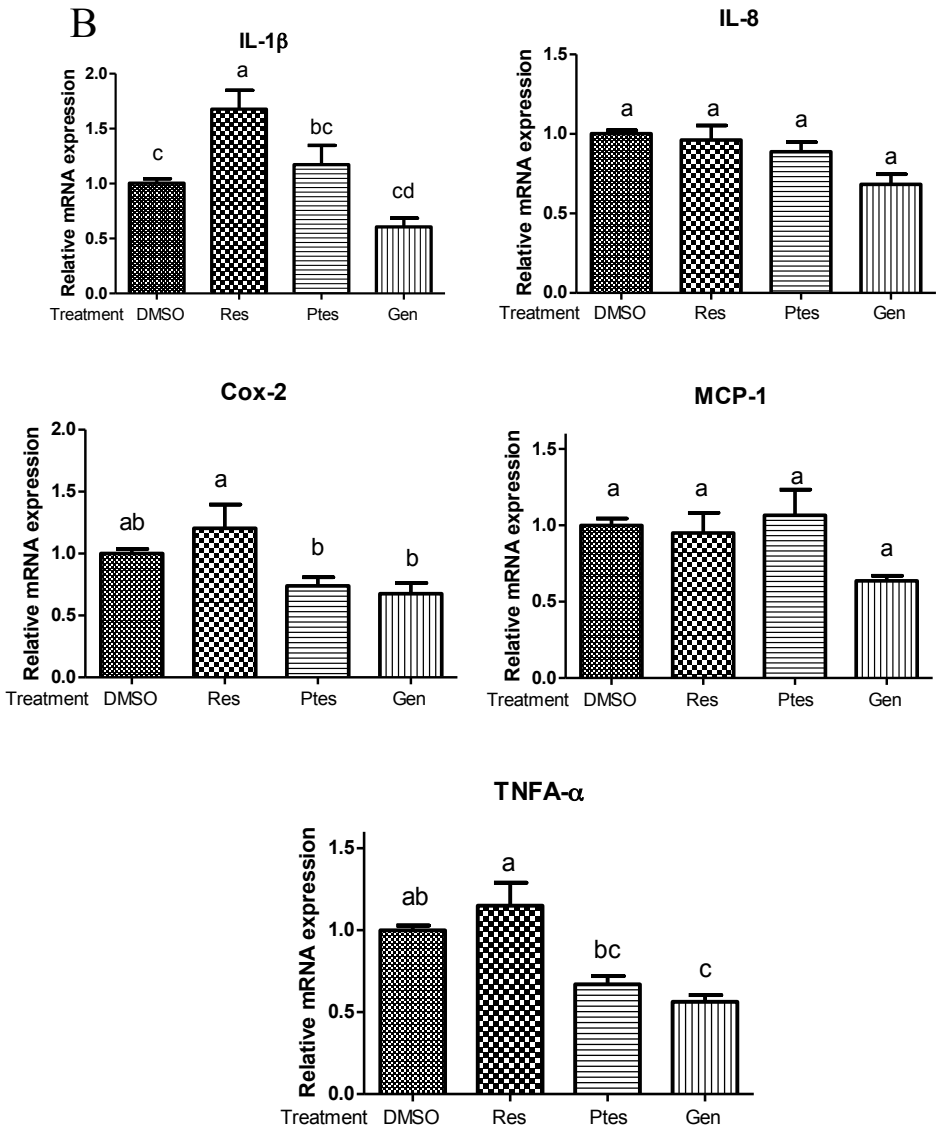


Fig. 11B

Effect of bioactive compounds on pro-inflammatory cytokine expression in P-THP-1 macrophages after LPS stimulation

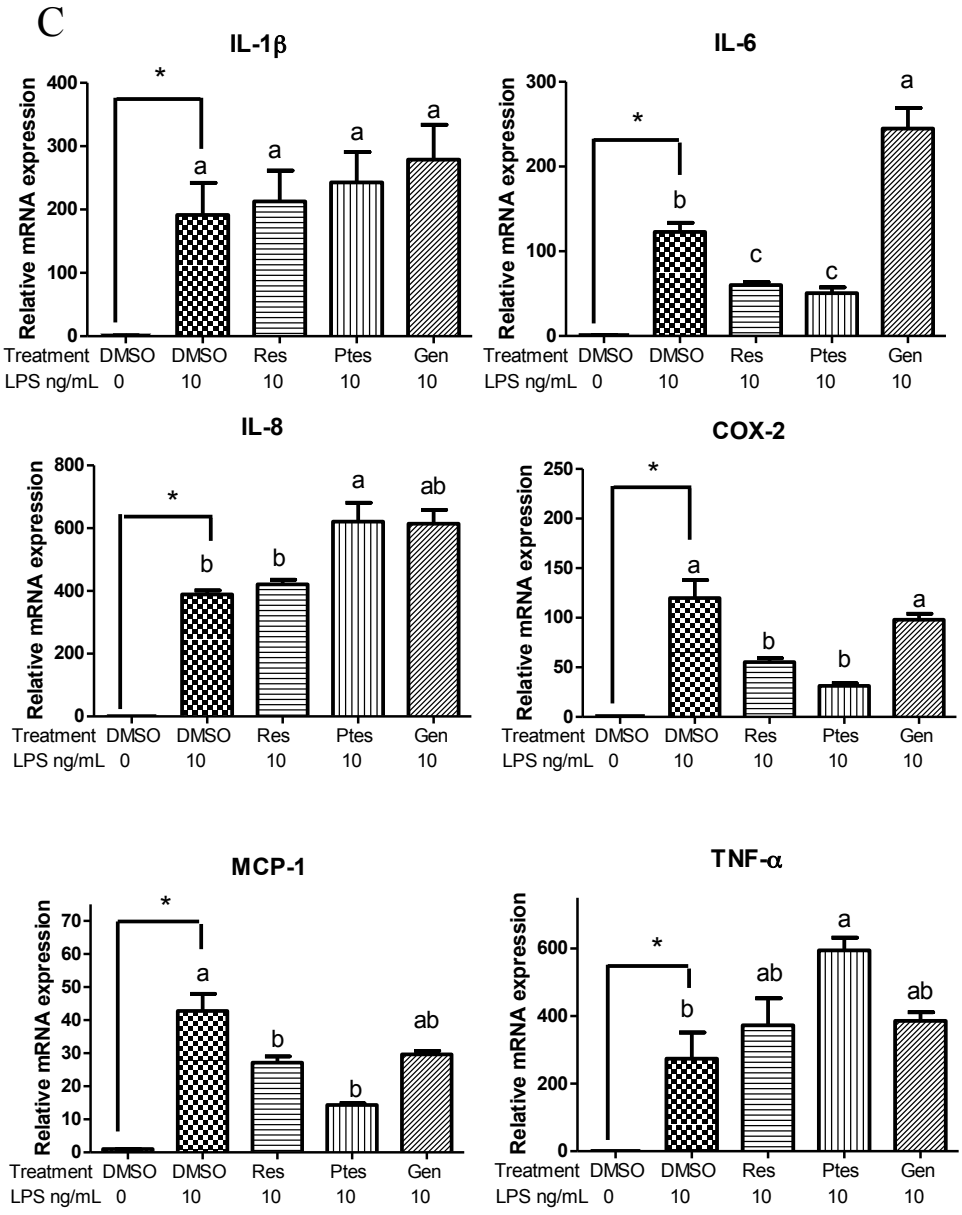


Fig. 11C

Effect of bioactive compounds on pro-inflammatory cytokine expression in P-THP-1 macrophages with no LPS stimulation

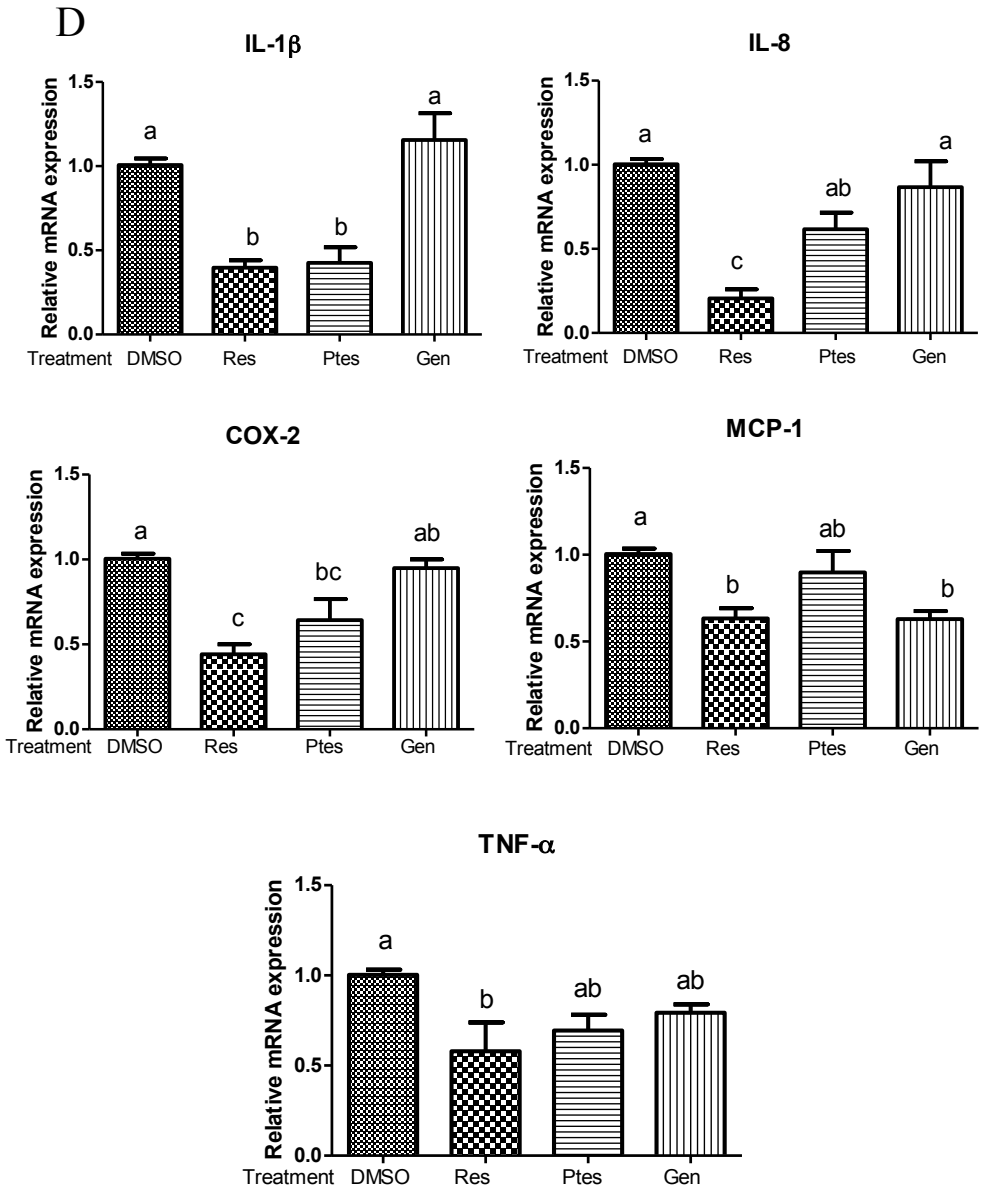


Fig. 11D

Figure 11. The effect of resveratrol, pterostilbene and genistein on pro-inflammatory gene expression in THP-1 monocytes and P-THP-1 macrophages with or without LPS stimulation. THP-1 monocytes (2.5×10^5/mL) were cultured overnight and then treated with DMSO (control), resveratrol (10 μM), pterolstilbene (10 μM) or genistein (10 μM) for 48 hours. Cells were then stimulated with 10 ng/mL LPS (A) or control-no LPS (B) for 2 hours at 37 °C. P-THP-1 macrophages (5×10^5/mL) were treated with DMSO (control), resveratrol (10 μM), pterolstilbene (10 μM) or genistein (10 μM) for 48 hours. Cells were then stimulated with 10 ng/mL LPS (C) or control-no LPS (D) for 2 hours at 37 °C. Cytokine mRNA expression of treated cells was measured by using quantitative RT-PCR. Values were expressed as the mean ± SE of 3 separate experiments. Means with different letters were significantly different ($p<0.05$). * was used to indicate means significantly different from control ($p<0.01$).

3.3.6 Effect of Resveratrol on the expression of IL-6 protein levels

LPS and PAM stimulation in THP-1 monocytes increased IL-6 protein level as compared to controls (LPS/PAM 0 ng/mL) (Fig.12A and B). IL-6 protein level, in LPS or PAM treated THP-1 cells (Fig.12A and B), increased progressively with Res treatment of Res 5 to 25 μM, or Res 10 to 25 μM, respectively.

Similarly, LPS or PAM stimulation of P-THP-1 macrophages markedly elevated IL-6 protein level as compared to controls (LPS/PAM 0 ng/mL) (Fig. 12D and E). However, IL-6 protein level in LPS or PAM treated P-THP-1 cells (Fig. 12D and E) decreased from Res 10 to 25 μM or Res 5 to 25 μM, respectively.

In bioactive compounds treated and LPS stimulated THP-1 monocytes (Fig. 12C) or P-THP-1 macrophages (Fig. 12F), IL-6 protein level was markedly increased or mildly depressed, respectively, by Res or Ptes treatments. Gen treatment induced no changes in these cells.

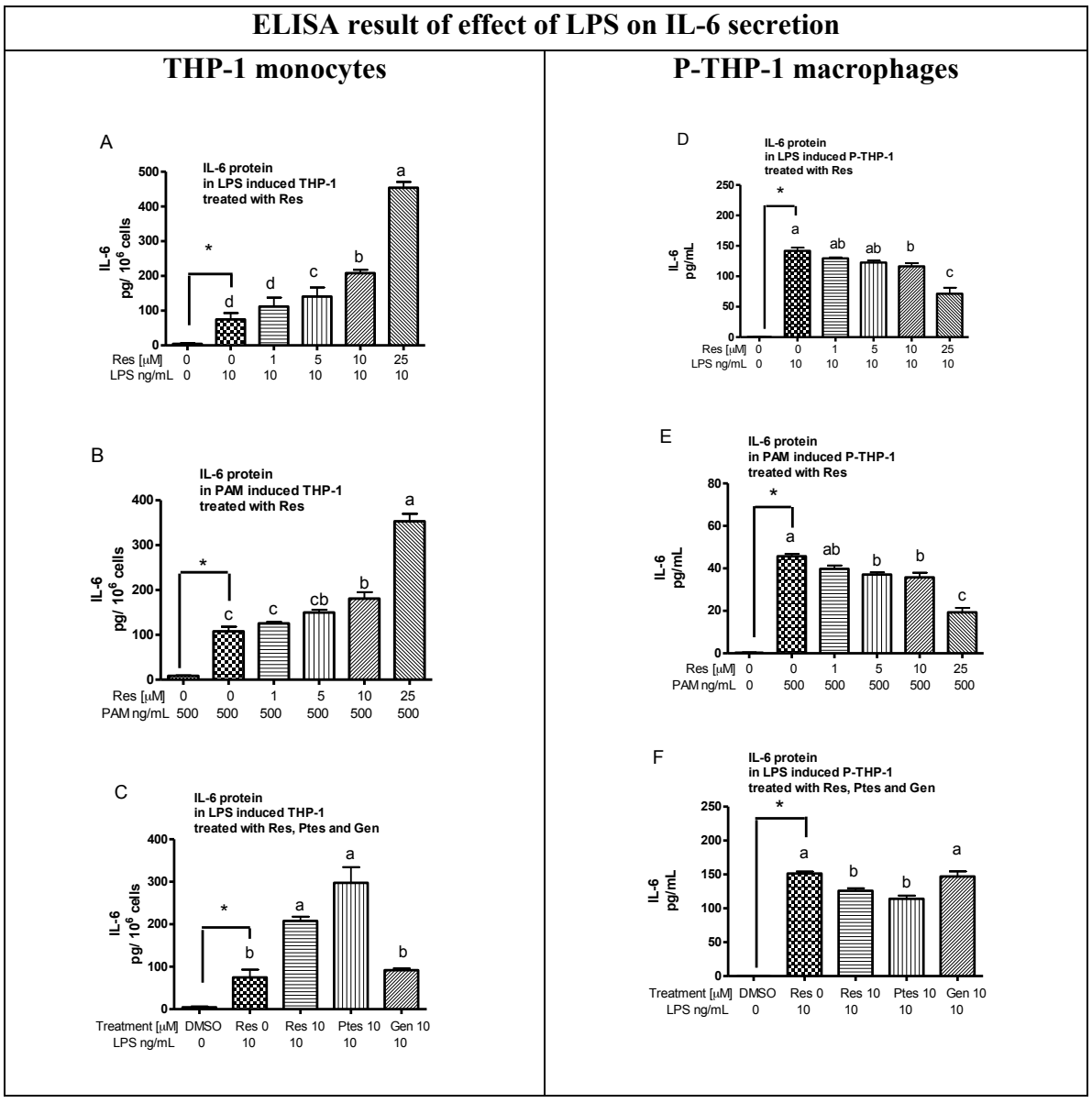


Figure 12. Effect of LPS on IL-6 secretion in THP-1 monocytes and P-THP-1 macrophages. THP-1 monocytes (A, B, C) and P-THP-1 macrophages (D, E, F) were pretreated with resveratrol for 48 h and then stimulated with LPS for 4 h and 5 h, respectively. IL-6 secretion was measured by using an ELISA. Data were normalized by cell count. Bars represent the mean ± SE of three independent experiments. Means with different letters were significantly different ($P < 0.05$).

3.4 Discussion

In **chapter 2**, we have discussed the effect of Res on THP-1 monocytes, we found that Res showed low IC50 in THP-1, inhibited the cell proliferation by elevating p21 mRNA and protein gene expression, and induced S phase and G0/G1 phase arrest with the Res levels (5 -10 μM), attainable by dietary consumption, and with pharmaceutical level (25 μM), respectively.

Hereinbelow, the effect of Res in the two cell lines, THP-1 and P-THP-1 cells, will be further discussed to determine whether Res has cell type specificity. THP-1 monocytes and P-THP-1 macrophages, derived from cancer cell lines, which are major cell types that play key role in chronic inflammation and also are involved in cancer, atherosclerosis and diabetes [114]. In our study, the influence of Res on cell proliferation in THP-1 and P-THP-1 cells, CYPs gene expression, pro-inflammatory cytokines and mediators' gene expression was examined to determine its effect related to cancer prevention and chronic inflammation. Data generated from these in vitro human cell-line studies will aid in the design of future studies involving normal cell types and animals.

To further study the relationship between Res structure and its effect, another stilbene structure compound, pterostlbene (Ptes), together with a non-stilbene compound, genistene (Gen), were used in a comparative study.

Firstly, we examined the influence of Res concentration, ranging from dosages attainable by diet to therapeutic level, on THP-1 and P-THP-1 cells. Although P-THP-1 macrophages were differentiated from THP-1 monocytes, they reacted differently from THP-1 monocytes. In other studies with cancer cell lines, Res induced variable effects on apoptosis and growth. In 2002, Andrew *et al*., [5858] reported that IC50 values for

apoptosis ranged from 70 to 150 μM in five human cancer cell lines (MCF7, SW480, HCE7, Seg-1 and HL60). Similarly, Helene *et al*., (2002) reported that apoptosis was induced in their Res treated lymphoid and myeloid leukemia cells with IC50 5-43 μM [59, 60, 61]. In contrast, apoptosis was undetected in Res treated HL60 cells (Human promyelocytic leukemia cells) [62, 63]. Although the culture conditions collectively were different among these studies and the present studies, the available data showed that Res acted differently on cell proliferation and apoptosis dependent on cell types. Our results showed that IC50 for THP-1 monocytes and P-THP-1 macrophages treated with Res was 17 and 76 μM, respectively, indicating that monocytes are much more sensitive than macrophages to resveratrol treatment.

Data from this study also indicate that Res at levels, attainable by dietary intake, did not show toxicity and apoptosis in either cell line. However, Res at 25 μM induced apoptosis in THP-1 leukemia cells, but not in P-THP-1 macrophages. Lower concentrations of Res (< 10 μM), attainable by usage of dietary supplements intake, did not cause any apoptosis and toxicity on THP-1 and P-THP-1 cells. As shown in other studies, THP-1 is sensitive to cytotoxic effect of Res [115], whereas P-THP-1 macrophages are refractory to Res [116], which are consistent with our result. From cell cycle results derived from THP-1 cells, Res induced anti-proliferation effect on cell growth with different cell cycle phase arrest in a dose-dependent manner. The effects of Res on cell cycle are clearly variable depending on the concentration of Res. At lower dosages, Res (5 and 10 μM) just induced S-phase arrest by inhibiting the proliferation of THP-1 without apoptosis. However, Res at 25 μM appeared to be toxic, and induced G0/G1 phase arrest and apoptosis in THP-1 monocytes.

P450 (CYP) cytochromes are enzymes that catalyze phase-I metabolism reactions, in which CYP1A1 and CYP1B1 are key members participating in the metabolisms of xenobiotics and endobiotics. CYPs, have oxygenase activity and commonly catalyze redox reactions, which is involved in the oxidation of the substrate and reduction of water. P450 1A1 (CYP1A1) catalyzes such reaction that involves in the hydroxylation of a vacant position of an aromatic ring on various drugs, food components or environment contaminants. Similar to CYP1A1, P450 1B1 (CYP1B1) activates several mutagens, like catalyzing the 4-hydroxylation of estrogens [117]. The two enzymes are also involved in the generation of superoxide radicals [118] and their expressions are enhanced in a diversity of tumor cells [119]. Reactions catalyzed by such enzymes are considered to initiate carcinogenic pathways and lead to cancer development. Inhibition of CYP1A1 and CYP1B1 by Res was shown in several studies [120, 121, 122]. In our study, CYP1A1 was not modulated by Res in THP-1 cells, and was not detected in P-THP-1 cells. Interestingly, CYP1B1 mRNA expression was decreased in both cell lines by Res 5 and 25 μM. In 2009, Beedanagari *et al*., [123] reported that 10 μM Res inhibited dioxin-induced expression of CYP1A1 and CYP1B1 at the transcription level in both human breast cancer cell line MCF-7 and human hepatocellular carcinoma cell line HepG2. Such results have also been confirmed by the research of Ciolino *et al.*, [122]. In contrast, our present study demonstrated that Res inhibited CYP1A1-independent CYP1B1 mRNA expression in both THP-1 and P-THP-1 cells, which indicated that Res can impair cancer development in THP-1 and P-THP-1 cells by down-regulating CYP1B1 mRNA expression. In addition, together with data found in other research, Res induced dose- and cell-type-dependent modification on CYP1A1 and CYP1B1 gene regulations.

In addition, Res gradually induced several pro-inflammatory cytokines gene expression in LPS-induced THP-1, which is different from the findings of Oh *et al.,* [124] that Res (0.1-100 μM) inhibited IL-8 production in LPS-induced THP-1 cells. However, the culture and treatment conditions used in their research were different from the present study and this may explain the conflicting findings. Oh *et al.,* [124] pre-treated THP-1 cells with Res for only 30 minutes, and then LPS were added for 24 hours together with Res. The effect of Res on THP-1 cells was too short, and LPS treatment was too long to examine the IL-8 mRNA expression because LPS-induced IL-8 transcripts in THP-1 cells have been reported to have a significantly shorter half-life (1.2 h) [125]. Moreover, Wang *et al.,* [126] found that at first resveratrol delayed LNCaP tumor growth and inhibited expression of a marker for steroid hormone responses in xenograft mice. However, resveratrol exposure for longer time led to increased angiogenesis and inhibition of apoptosis in the xenograft. Wang's [126] report suggested that Res might delay some genes transcription expression and induced the cancer promotion later. Hence, together with our result that Res enhance pro-inflammatory cytokines mRNA expression in THP-1 cells on a concentration-dependent manner to enhanced inflammation, Res may have a delay effect on pro-inflammatory cytokines secretion. However, since Res has low IC50 (17 μM), 48-hour treatment of Res with increasing concentrations may induce pro-inflammation effect due to its possible toxic effect on the cells, which may be regarded as a harmful stress.

On the other hand, the findings that Res can have such variable effects between THP-1 and P-THP-1 cells and that Res treatment induces anti-inflammation effect in P-THP-1 macrophages is very interesting. Based on our results, Res appeared to show less

harm for the growth of P-THP-1 macrophages and induced inhibition on selected pro-inflammatory cytokines with or without inflammation induction by LPS or PAM. Under stimuli (LPS or PAM), NF-κB pathway is responsible for the enhanced production of pro-inflammatory cytokines [127]. These findings suggest that Res, in a dose-dependent manner, can inhibit inflammation effect by partially reducing pro-inflammatory cytokines, MCP-1 and COX-2 in P-THP-1 macrophages.

Interestingly, In LPS/PAM stimulated P-THP-1 macrophages, Res at 10 and 25 μM increased TNF-α mRNA levels, which were different from the rest of the findings that Res inhibited IL-1β, IL-6 and IL-8 mRNA expression or did not show any effect. Inconsistent observation of TNF-α mRNA expression may be caused by several reasons. One is that the treatment time on LPS/PAM stimulated P-THP-1 macrophages did not show the peak mRNA expression of TNF-α expression, but showed an opposite expression trend. Another reason is that Res may trigger different pathway of TNF-α, IL-1β, IL-6 and IL-8. Alternatively, Res may have up-regulated promoter gene of TNF-α mRNA and increased its expression in P-THP-1 cells. Our findings on the expression of the 4 genes in the LPS/PAM stimulated P-THP-1 cells provide limited data, and the inconsistent outcome needs further investigation on such signaling pathway.

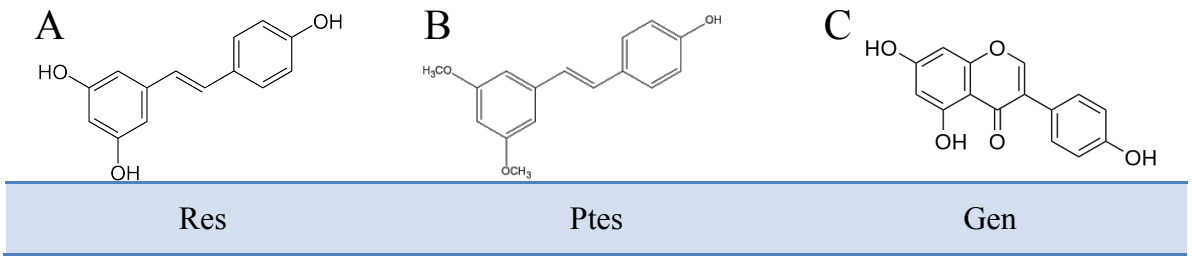


Figure 13. Structure of resveratrol-Res (A), pterostilbene-Ptes (B) and genistein-Gen (C).

By observing the effects of Res on THP-1 and P-THP-1 cell lines, we then investigated whether or not the effect of Res is derived from the stilbene structure. To further confirm this hypothesis, one stilbene-based compound, pterostilbene (Fig. 13B), and a non-stilbene-based compound, genistein (Fig. 13C), were selected for our study. From the pro-inflammatory cytokine mRNA expression results, Ptes was shown to exert similar effect as Res, whereas Gen showed no effects. Data from these studies suggested that stilbene-structure compounds exerted anti-inflammation function in P-THP-1 macrophages, with or without LPS stimulation, and induced inflammation in LPS-induced THP-1 cells.

3.5 Conclusion:

In the present study, we aim to determine whether the effects of Res have cell type specificity, and whether the basic stilbene structure accounts for inducing such effects. Results from the present study showed that Res exerted anti-proliferation in both cell lines, and possibly anti-cancer function by elevating p21 gene expression and down-regulating CYP1B1 mRNA level on THP-1 monocytes, as well as cytokine-selected pro or anti-inflammation effects on THP-1 or P-THP-1 cells, respectively. By further comparison with two other structure-specific compounds, both Res and Ptes showed similar effects. Our conclusion is that resveratrol exhibited cell-type- and concentration-dependent toxicity, exhibited blocking cancer development property by down regulation of CYP1B1 in both cell lines, and exerted pro- or anti-inflammation effect on monocytes or macrophages, respectively. Results from the present study support the hypothesis that Res showed different effects on different cell types, and such effects are attributed to the basic stilbene structure.

Chapter 4 Future study

Although resveratrol has been studied for more than two decades, its effects on innate immune cells under physiologic attainable conditions are seldom examined. In our study, our first aim is to determine the effect of Res (from dietary to pharmaceutical concentrations) in THP-1 monocytes regarding the toxicity and cancer prevention function. In our study, Res exhibited concentration-dependent toxicity, induced different cell cycle arrest on a concentration manner, exhibited blocking of cancer development property by inducing p53-independent p21 gene expression, which is a possible candidate for the potential cancer treatment.

In the present study, we found that resveratrol exhibited cell-type- and concentration-dependent toxicity, exhibited blocking cancer development by down regulation CYP1B1 in both cell lines, and exerted pro- or anti-inflammation on monocytes or macrophages, respectively.

Currently, we have examined the effect of Res with physiological attainable concentrations in human THP-1 monocytes and its derived P-THP-1 macrophages with Res. In the future, the long-term effects of daily consumption of Res can be further examined. In addition, normal cell lines can be used in more precise and applicable studies. These will then lead to animal and human studies on the effects of long-term Res consumption, which will generate scientific evidence needed for the establishment of guidelines of Res consumption as dietary supplements or medicine.

REFERENCES:

[1] Celsus. De medicina. Spencer WG. Heinemann, London 1935.
[2] Ferrero-miliani, L., Nielsen, O. H., Andersen, P. S., Girardin, S. E., Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. *Clin. Exp. Immunol.*2007, *147*, 227-235.
[3] Tabas, I., Glass, C. K., Anti-inflammatory therapy in chronic disease: challenges and opportunities.*Science*2013, *339*, 166-72.
[4] Doherty, D. E., Downey, G. P., Worthen, G. S., Haslett, C., Henson, P. M., Monocyte retention and migration in pulmonary inflammation required for neutrophils.*Lab Invest.* 1998, *59*, 200-213.
[5] Kindt,T. J., Osborne, B. A., Goldsby, R, A.,(6 edition), *Kuby Immunology*. W. H. Freeman &Company,2006, pp. 4-10.
[6] Janeway, C. A. Jr., Travers, P., Walport, M., et al. (5th edition), *Immunology: the immune system in health and disease*,Garland Science,New York, 2001.
[7] Akira S., Uematsu S., Takeuchi O., Pathogen recognition and innate immunity.*Cell*2006, *124*, 783-801.
[8] Akira, S., Takeda, K., Toll-like receptor signaling. *Nat. Rev. Immuno.*2004, *4*, 499-511.
[9] Brocker, C., Thompson, D., Matsumoto, A., Nebert, D.W., Vasiliou, V., Evolutionary divergence and functions of the human interleukin (IL) gene family. *Human Genomics* 2010, *5*, 30–55.
[10] Gabay, C., Interleukin-6 and chronic inflammation. *Arthritis research and therapy* 2006, *8* (Suppl 2): S3.
[11] Sun, M., Fink, P.J., A new class of reverse signaling costimulators belongs to the TNF family. *J. Immunol*. 2007, *179*, 4307–12.
[12] Kriegler, M., Perez, C., DeFay, K., Albert, I., Lu, S. D., A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* 1998, *53*, 45–53.
[13] Parham, Peter., *Chapter 6: T-cell mediated immunity-The immune system (2nd ed.),* Garland Science, New York 2005, pp.172.
[14] Hussain, S. P., Raja, K., Amstad, P. A., Sawyer, M.,Trudel, L. J., Wogan, G. N., Hofseth, L. J., Shields, P. G., Billiar, T. R., Trautwein, C., Hohler, T., Galle, P. R., Increased p53mutation load in nontumorous human liver of Wilson disease and hemochromatosis: oxyradical overload diseases. *Proc. Natl. Acad. Sci. USA*. 2000, *97*, 12770-5.
[15] Lekli, I., Ray D., Das, D. K., Longevity nutrients resveratrol, wines and grapes. *Genes Nutr.* 2010, *5*, 55–60.

[16] Baur, J. A., Sinclair, D. A. Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discov.* 2006, *5*, 493–506.
[17] Das, S., Das, D. K., Wine and the heart: a journey from grapes to resveratrol. *S. Afr. J. Enol. Vitic.* 2006, *27*, 127–132.
[18] Romero-Pérez, A. I., Ibern-Gómez, M., Lamuela-Raventós, R. M., de La Torre-Boronat, M. C., Piceid, the major resveratrol derivative in grape juices. *J. Agric. Food Chem.* 1999, *47*, 1533-6.
[19] Counet, C., Callemien, D., Collin, S., Chocolate and cocoa: New sources of trans-resveratrol and trans-piceid. *Food Chemistry* 2006, *98*, 649-657.
[20] Stervbo, U., Vang, O., Bonnesen, C., A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine. *Food Chemistry* 2007, *101*, 449-457.
[21] Langcake, P., Pryce, R. J., A new class of phytoalexins from grapevines. *Experientia.* 1977, *33*, 151.
[22] Scarlatti, F., Sala, G., Somenzi, G., Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells via de novo ceramide signaling. *FASEB J.* 2003, *17*, 2339–2341.
[23] Schubert, R., Fischer, R., Hain, R., Schreier, P. H., Bahnweg, G., Ernst, D., Sandermann, H. Jr., An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-responsive sequence. *Plant Mol. Biol.* 1997, *34*, 417-426.
[24] Aziz, M. H., Reagan-Shaw, S., Wu, J., Longley, B. J., Ahmad, N. Chemoprevention of skin cancer by grape constituent resveratrol: relevance to human disease? *FASEB J.* 2005, *19*, 1193–1195.
[25] Bhat, K. P., Pezzuto, J. M., Cancer chemopreventive activity of resveratrol. *Ann. N. Y. Acad. Sci.* 2002, *957*, 210–229.
[26] Shankar, S., Singh, G., Srivastava, R. K., Chemoprevention by resveratrol: molecular mechanisms and therapeutic potential. *Front. Biosci.* 2007, *12*, 4839–4854
[27] Li, F., Gong, Q., Dong, H., Shi, J. Resveratrol, a neuroprotective supplement for Alzheimer's disease. *Curr. Pharm. Des.* 2012, *18*, 27-33.
[28] Soleas, G. J., Yan, J., Goldberg, D. M., Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. *J. Chromatogr. B. Biomed. Sci. Appl.* 2001, *757*, 161–172.
[29] Goldberg, D. M., Yan, J., Soleas, G. J., Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin. Biochem.* 2003, *36*, 79–87.

[30] Meng, X., Maliakal, P., Lu, H., Lee, M. J., Yang, C. S., Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. *J. Agric. Food Chem.* 2004, *52*, 935–942.
[31] Burkon, A., Somoza, V., Quantification of free and proteinbound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides – two novel resveratrol metabolites in human plasma. *Mol. Nutr. Food Res.* 2008, *52*, 549–557.
[32] Boocock, D. J., Patel, K. R., Faust, G. E., Normolle, D. P. et al., Quantitation of trans-resveratrol and detection of its metabolites in human plasma and urine by high performance liquid chromatography. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2007, *848*, 182-187.
[33] Boocock, D. J., Faust, G. E., Patel, K. R., Schinas, A. M. et al., Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiol. Biomarkers Prev.* 2007, *16*, 1246-1252.
[34] Cottart, C. H., Nivet-Antoine, V., Laguillier-Morizot, C., Beaudeux, J. L., Resveratrol bioavailability and toxicity in humans. *Mol. Nutr. Food Res*. 2010, *54*, 7-16.
[35] Stroniolo, C. E., Moreno, J. J., Resveratrol metabolites have an antiproliferative effect on intestinal epithelial cancer cells. *Food Chemistry* 2012, *134*, 1385-1391.
[36] Johnson, J. J., Nihal, M., Siddiqui, I. A., Scarlett, C. O., Bailey, H. H., Mukhtar, H., Ahmad, N., Enhancing the bioavailability of resveratrol by combining it with piperine. Mol. Nutr. *Food Res.* 2011, *55*, 1169-76.
[37] La Porte, C., Voduc, N., Zhang, G., Seguin, I., Tardiff, D., Singhal, N., Cameron, D. W., Steady-State pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. *Clin. Pharmacokinet.* 2010, *49,* 449-454.
[38] Elliott, P. J., Walpole, S., Morelli, L., Lambert, P. D., Lunsmann, W., Westphal, C. H., Lave, S., Resveratrol/SRT501. *Drugs Fut.* 2009, *34*, 291-295.
[39] Crowell, J. A., Korytko, P. J., Morrissey, R. L., Booth, T. D., Levine, B. S., Resveratrol-associated renal toxicity. *Toxicol. Sci.* 2004, *82*, 614-619.
[40] Renaud, S., de Lorgeril, M., Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 1992, *339*, 1523–6.
[41] Richard, J. L., Cambien, F., Ducimetie`re, P., Epidemiologic characteristics of coronary disease in France. *Nouv. Presse. Med.* 1981, *10*, 1111-4.
[42] Artaud-Wild, S. M., Connor, S. L., Sexton, G., Connor W. E., Differences in coronary mortality can be explained by differences in cholesterol and saturated fat intakes in 40 countries but not in France and Finland. A paradox. *Circulation* 1993, *88*, 2771–9.
[43] Gronbaek, M., Becker, U., Johansen, D., Gottschau, A., Schnohr, P., Hein, HO., Hensen, G., Sørensen, TI., Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. *Ann. Intern. Med.* 2000, *133*, 411–9.
[44] Cabot, R.C., The relation of alcohol to atherosclerosis. *JAMA* 2004, *43*, 774-775.

[45] Inhibition of LDL oxidation by phenolic substances in red wine: a clue to the French paradox. *Nutr Rev.* 1993, *51*, 185-7.
[46] Murphy, S. L., Xu, J. Q., Kochanek, K. D., QuickStats: Number of Deaths from 10 Leading Causes. *National Vital Statistics System, United States*, 2010.
[47] Kravchenko, J., Akushevich, I., Manton, K. G., *Cancer mortality and morbidity patterns in the U. S. population: an interdisciplinary approach*, Springer, Berlin 2009.
[48] Silverstein A., Silverstein, V. B., Nunn, L. S., *Cancer*, Conn: Twenty-First Century Books, Brookfield 2006, pp. 11-12.
[49] Strayer D. L., Rubin R., Emanuel R., *Rubin's pathology: clinicopathologic foundations of medicine*, Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia 2008, pp. 138-139.
[50] Elledge, S. J., Cell cycle checkpoints: prevention an identity crisis. *Science* 1996, *274*, 1664-72.
[51] Morgan, D. O., The cell cycle: principles of control (1st edition), New Science Press, London 2007.
[52] Lodish, H., Berk, A., Matsudaira, P., et al., Molecular cell biology (5th edition), WH Freeman, New York 2004.
[53] Gali-Muhtasib, H., Bakkar, N., Modulating cell cycle: current applications and prospects for future drug development. *Curr. Cancer Drug Targets* 2002, *2*, 309-336.
[54] Kundu, J. K., Surh, Y. J., Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. *Cancer Lett.* 2008, *269*, 243-61.
[55] Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., Zappia, V., Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem. Biophys. *Res. Commun.* 1998, *250*, 53-8.
[56] Park, J. W., Choi, Y. J., Jang, M. A., Lee, Y. S., Jun, D. Y., Suh, S. I., Baek, W. K., Suh, M. H., Jin, I. N., Kwon, T. K., Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. *Cancer Lett.* 2001, *163*, 43-9.
[57] Tsan, M. F., White, J. E., Maheshwari, J. G., Chikkappa, G., Anti-leukemia effect of resveratrol. *Leuk. Lymphoma* 2002, *43*, 983-7.
[58] Joe, A. K., Liu, H., Suzui, M., Vural, M. E., Xiao, D., Weinstein, I. B., Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin. Cancer Res.* 2002, *8*, 893-903.
[59] Ferry-Dumazet, H., Garnier, O., Mamani-Matsuda, M., Vercauteren, J., Belloc, F., Billiard, C., Dupouy, M., Thiolat, D., Kolb, J. P., Marit, G., Reiffers, J., Mossalayi, M. D., Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells. *Carcinogenesis* 2002, *23*, 1327-33.

[60] Tsan M.F., White J.E., Maheshwari J.G., Bremner T.A., Sacco J., Resveratrol induces Fas signaling-independent apoptosis in THP-1 human monocytic leukemia cells. *British journal of Haematology* 2000, *109*, 405-412.
[61] Bernhard, D., Tinhofer, I., Tonko, M., Hubl, H., Aysserlechner, M.j., Greil, R., Kofler, R., Csordas, A., Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukemia cells. *Cell death and differentiation* 2000, *7*, 834-842.
[62] Ragione F.D., Cucciolla V., Borriello A. Pietra V.D., Racioppi L., Soldati G., Manna C., Galletti P., Zappia V., Resveratrol arrests the cell division cycle at S/S2 phase transition. *Biochemical biophysical research communication* 1998, *250*, 53-58.
[63] Surl, Y.J., Hurh, Y.J., Kang, J.Y., Lee, E., Kong, G., Lee, S.j., Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL60) cells. *Cancer letters* 1999, *249*, 109-115.
[64] Gomez-Angelats, M., Bortnor, C. D., Cidlowski, J. A., Protein kinase C (PKC) inhibits Fas receptor- induced apoptosis through modulation of the loss of K+ and cell shrinkage. *J. Biol. Chem*. 2000, *275*, 19609-19619.
[65] Schoene, N. W., Kelly, M. A., Polansky, M.M., Anderson, R.A., Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines. *Cancer Lett.* 22005, *30*, 134-40.
[66] Krutzik, P. L., Nolan, G. P., Intracellular phosphor-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A.* 2003, *55*, 61-70.
[67] Chow, S., Hedley, D., Grom, P., Magari, R., Jacobberger, J. W., Shankey, T. V., Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. *Cytometry A*. 2005, *67*, 4-17.
[68] Akashi, M., Osawa, Y., Koeffler, H. P., Hachiya, M., p21WAF1 expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: important role of RNA stabilization. *Biochem. J.* 1999, *337*, 607-16.
[69] Lee, M. H., Reynisdóttir, I., Massagué, J., Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev*. 1995, *9*, 639-49.
[70] Todd, I.,*Cells of the immune system*, Encyclopedia of life science, 2001.
[71] Michael, K., Florian, R. G., NF-κB: linking inflammation and immunity to cancer development and progression. *Nature review immunology* 2005, *5,* 749-759.
[72] Glenn, D., Cytokines in cancer pathogenesis and cancer therapy. *Nature reviews cancer* 2004, *4*, 11-22.
[73] Hussain, S. P., Raja, K., Amstad, P. A., Sawyer, M., Trudel, L.J., Wogan, G. N., Hofseth, L. J., Shields, P. G., Billiar, T. R., Trautwein, C., Hohler, T., Galle, P. R., Increased p53mutation load in nontumorous human liver of Wilson disease and

hemochromatosis: oxyradical overload diseases. *Proc. Natl. Acad. Sci. USA* 2000, *97*, 12770-5.
[74] Hussain, S. P., Harris, C. C., Inflammation and cancer: an ancient link with novel potentials. *Int. J. Cancer* 2007, *121*, 2373-2380.
[75] Espey, M. D., Miranda, K. M., Feelisch, M., Fukuto, J., Grisham, M. B., Vitek, M. P., Wink, D. A. , Mechanisms of cell death governed by the balance between nitrosative and oxidative stress. *Ann. N. Y. Acad. Sci.* 2000, *899*, 209-21.
[76] Hussain, S. P., Amstad, P., Raja, K., Ambs, S., Nagashima, M., Bennett, W. P., Shields, P. G., Ham, A. J., Swenberg, J. A., Marrogi, A. J., Harris, C. C., Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res.* 2000, *60*, 3333-7.
[77] Dinarello, C. A., Proinflammatory cytokines. *Chest.* 2000, *118*, 503-8.
[78] Dinarello, C. A., The paradox of pro-inflammatory cytokines in cancer. *Cancer Metastasis Rev.* 2006, *25*, 307-313.
[79] Balkwill, F., Charles, K. A., Mantovani, A., Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005, *7*, 211–217.
[80] Correa P., Piazuelo M. B., Natural history of Helicobacter pylori infection. *Dig. Liver Dis.* 2008, *40*, 490–6.
[81] Fock, K. M., Ang, T. L., Epidemiology of Helicobacter pylori infection and gastric cancer in Asia. *J. Gastroenterol Hepatol.* 2010, *25*, 479-86.
[82] Chang, Y. W., Jang, J. Y., Kim, N. H., Lee, J. W., Lee, H. J., Jung, W. W., Interleukin-1B (IL-1B) polymorphisms and gastric mucosal levels of IL-1beta cytokine in Korean patients with gastric cancer. *International Journal Of Cancer* 2005, *114*, 465-471.
[83] Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth H., Turner L., Rollins B., Pasparakis M., Kollias G., Balkwill F., Mice deficient in tumor necrosis factor-a are resistant to skin carcinogenesis. *Nat. Med.* 1999, *5*, 828–31.
[84] Hilbert, D. M., Kopf , M., Mock, B.A., Kohler, G., Rudikoff, S., Interleukin 6 is essential for in vivo development of B lineage neoplasms. *J. Exp. Med.* 1995, *182*, 243–8.
[85] Lattanzio, G., Libert, C., Aquilina, M., Cappelletti, M., Ciliberto, G., Musiani, P., Poli, V., Defective development of pristane-oil-induced plasmacytomas in interleukin-6-deficient BALB/c mice. *Am. J. Pathol.* 1997, *151*, 689–96.
[86] Smith, D. R., Polverini, P. J., Kunkel, S. L., Orringer, M. B., Whyte, R. I., Burdick, M. D., Wilke, C. A., Strieter, R. M., Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. *J. Exp. Med.* 1994, *179*, 1409–15.
[87] Yao, P. L., Lin, Y. C., Wang, C. H., Huang, Y. C., Liao, W. Y., Wang, S. S., Chen, J. J., Yang, P. C., Autocrine and paracrine regulation of interleukin-8 expression in lung cancer cells. Am. J. *Respir. Cell Mol. Biol.* 2005, *32*, 540–7.
[88] Vincent, T. , Jourdan, M., Sy, M. S., Klein, B., Mechti, N., Hyaluronic acid induces survival and proliferation of human myeloma cells through an interleukin-6-mediated

pathway involving the phosphorylation of retinoblastoma protein. *J. Biol. Chem*. 2001, *276*, 14728–36.
[89] Miyamoto, Y., Hosotani, R., Doi, R., Wada, M., Ida J., Tsuji, S., Kawaguchi, M., Nakajima, S., Kobayashi, H., Masui, T., Imamura, M., Interleukin-6 inhibits radiation induced apoptosis in pancreatic cancer cells. *Anticancer Res.* 2001, *21*, 2449–56.
[90] Renner, P., Roger, T., Calandra, T., Macrophage migration inhibitory factor: gene polymorphisms and susceptibility to inflammatory diseases. *Clin. Infect. Dis.* 2005, *41* (Suppl 7): S513–S519.
[91] Karin, M., Nuclear factor-κB in cancer development and progression. *Nature* 2006, *441*, 431–6.
[92] Luo, J. L., Kamata, H., Karin, M., IKK/NF-κB signaling: balancing life and death—a new approach to cancer therapy. *J. Clin. Invest.* 2005, *115*, 2625–32.
[93] Araki, Y., Okamura, S., Hussain, S. P., Nagashima, M., He P., Shiseki, M., Miura, K., Harris, C. C., Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways. *Cancer Res.* 2003, *63*, 728–34.
[94] Tsujii, M., Kawano, S., DuBois, R. N., Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. *Natl. Acad. Sci.* 1997, *94*, 3336–40.
[95] Ridker, P. M., Cushman, M., Stampfer, M.J., Tracey, R. P., Hennekens, C. H., Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N. Engl. J. Med*. 1997, *336*, 973-979.
[96] Tracy, R. P., Lemaitre, R. N., Psaty, B. M., Ives, D. G., Evans, R. W., Cushman, M., Meilahn, E. N., Kuller, L. H., Relationship of C-reactive protein to risk of cardiovascular disease in the elderly. Results from the Cardiovascular Health Study and the Rural Health Promotion Project. *Arterioscler. Thromb. Vasc. Biol.* 1997 , *17*, 1121-7.
[97] Ridker, P. M., Buring, J. E., Shih J., Matias, M., Hennekens, C. H., Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation* 1998, *98*, 731-733.
[98] Kats, J., Marc, H., Porter, S., Ruskin, J., Inflammation, periodontitis, and coronary heart disease. *The lancet* 2001, *358*, 1998.
[99] Yu, W., Fu, Y. C., Wang, W., Cellular and molecular effects of resveratrol in health and disease. *Journal of cellular biochemistry* 2012, *113*, 752-759.
[100] Jang, J.H., Surh, Y.J., Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. *Mutat Res.* 2001, *496*, 181-190.
[101] Liu, J.C., Chen, J.J., Niu, X.F., et al., Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. *Hypertension* 2003, *42*, 1198-1205.
[102] Yu, W., Fu, Y.C., Zhou, X.H., et al. Effects of resveratrol on H2O2-induced apoptosis and expression of SIRTs in H9c2cells. *J. Cell Biochem.* 2009, *107*, 741-747.

[103] Zhong, M., Cheng, G. F., Wang, W. J., et al., Inhibitory effect of resveratrol on interleukin 6 release by stimulated peritoneal macrophages of mice. Phytomedicine 1999, *6*, 79-84.
[104] Rius, C., Abu-Taha, M., Hermenegildo, C., et al., Trans- but not cis-resveratrol impairs angiotensin-II-mediated vascular inflammation through inhibition of NF-kB activation and peroxisome proliferator-activated receptor-gamma upregulation. *J. Immunol.* 2010, *185*, 3718–27.
[105] Olholm, J., Paulsen, S.K., Cullberg, K.B., et al., Anti-inflammatory effect of resveratrol on adipokine expression and secretion in human adipose tissue explants. *Int. J. Obes.* 2010, *34*, 1546–53.
[106] Kundu, J.K., Surh, Y.-J., Breaking the relay in deregulated cellular signal transduction as a rationale for chemoprevention with anti-inflammatory phytochemicals. *Mutat. Res.* 2005, *591*, 123–146.
[107] Muller-Decker, K., Neufang, G., Berger, I., Neumann, M., Marks, F., Furstenberger, G., Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. Proc. *Natl. Acad. Sci. USA* 2002, *99*, 12483–12488.
[108] Tiano, H.F., Loftin, C.D., Akunda J., Lee, C.A., Spalding, J., Sessoms, A., Dunson, D.B., Rogan, E.G., Morham, S.G., Smart, R.C., Langenbach, R., Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis, *Cancer Res*. 2002, *62*, 3395–3401.
[109] Subbaramaiah, K., Chung, W.J., Michaluart P., et al., Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J. Biol. Chem.* 1998, *273*, 21875-21888.
[110] Kang, O. H., Jang, H. J., Chae, H. S., Oh, Y. C., Choi, J. G., Lee, Y. S., Kim, J. H., Kim, Y. C., Sohn, D. H., Park, H., Kwon, D.Y., Anti-inflammatory mechanisms of resveratrol in activated HMC-1 cells: pivotal roles of NF-kappaB and MAPK. *Pharmacol. Res.* 2009, *59*, 330-7.
[111] Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., Boyd, M. R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 1990, *82*, 1107-12.
[112] Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A., Boyd, M. R., Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J. Natl. Cancer Inst.* 1990, *82*, 1113-8.
[113] Park, E .K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C., Kim, K. S., Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm. Res.* 2007, *56*, 45-50.
[114] Needham, L. A., Davidson, A. H., Bawden, L. J., Belfield, A., Bone, E. A., Brotherton, D. H., Bryant, S., Charlton, M. H., Clark, V. L., Davies, S. J., Donald, A.,

Day, F. A., Krige, D., Legris, V., McDermott, J., McGovern, Y., Owen, J., Patel, S. R., Pintat, S., Testar, R. J., Wells, G. M., Moffat, D., Drummond, A. H., Drug targeting to monocytes and macrophages using esterase-sensitive chemical motifs. *J. Pharmacol. Exp. Ther.* 2001, *339*, 132-42.
[115] Feng, Y. H., Zhu, Y. N., Liu, J., Ren, Y. X., Xu, J. Y., Yang, Y. F., Li, X. Y., Zou, J. P., Differential regulation of resveratrol on lipopolysacchride-stimulated human macrophages with or without IFN-gamma pre-priming. *Int. Immunopharmacol.* 2004, *4*, 713-20.
[116] Tsan, M. F., White, J. E., Maheshwari, J. G., Bremner, T. A., Sacco, J., Resveratrol induces Fas signalling-independent apoptosis in THP-1 human monocyticleukaemia cells. *Br. J. Haematol*. 2000, *109*, 405-12.
[117] Guengerich, F., Chun, Y.J., Kim, D., Gillam, E.M., Shimada, T., Cytochrome P450 1B1: a target for inhibition in anticarcinogenesis strategies. *Mutat. Res.* 2003, *523–524*, 173–182.
[118] Buettner, G. R., Superoxide dismutase in redox biology: the roles of superoxide and hydrogen peroxide. *Anticancer Agents Med. Chem.* 2011, *11*, 341–346.
[119] Piotrowska, H., Kucinska M., Murias M., Expression of CYP1A1, CYP1B1 and MnSOD in a panel of human cancer cell lines. *Mol. Cell Biochem.* 2013, *383*, 95-102.
[120] Piver, B., Fer, M., Vitrac, X., Merillon, J. M., Dreano, Y., Berthou, F., Lucas, D., Involvement of cytochrome P450 1A2 in the biotransformation of trans-resveratrol in human liver microsomes. *Biochem. Pharmacol.* 2004, *68*, 773–782.
[121] Chow, H. H., Garland, L. L., Hsu, C. H., Vining, D. R., Chew, W. M., Miller, J.,A., Perloff, M., Crowell, J. A., Alberts, D. S., Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study. *Cancer Prev. Res. (Phila)*. 2010, *9*, 1168-75.
[122] Ciolino, H. P., Yeh, G. C., Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol. *Mol. Pharmacol.* 1999, *56*, 760-7.
[123] Beedanagari, S. R., Taylor, R. T., Bui, P., Wang, F., Nickerson, D. W., Hankinson, O., Role of epigenetic mechanisms in differential regulation of the dioxin-inducible human CYP1A1 and CYP1B1 genes. *Mol. Pharmacol*. 2010, *78*, 608-16
[124] Oh, Y. C., Kang, O. H., Choi, J. G., Chae, H. S., Lee, Y. S., Brice, O. O., Jung, H. J., Hong, S. H., Lee, Y. M., Kwon, D. Y., Anti-inflammatory effect of resveratrol by inhibition of IL-8 production in LPS-induced THP-1 cells. *Am. J. Chin. Med.* 2009, *37*, 1203-14.
[125] Harrison, L. M., van den Hoogen, C., van Haaften, W. C., Tesh, V. L., Chemokine expression in the monocytic cell line THP-1 in response to purified shiga toxin 1 and/or lipopolysaccharides. *Infect. Immun.* 2005, *73*, 403-12.

[126] Wang, T. T., Hudson, T. S., Wang, T. C., Remsberg, C. M., Davies, N. M., Takahashi, Y., Kim, Y. S., Seifried, H., Vinyard, B. T., Perkins, S. N., Hursting, S. D., Differential effects of resveratrol on androgen-responsive LNCaP human prostate cancer cells in vitro and in vivo. *Carcinogenesis* 2008, *29*, 2001-10.
[127] Chen, F., Kuhn, D. D., Sun, S.C., Gaydos, L.J., Demers, L.M., Dependence and reversal of nitric oxide production on NF-kappa B in silica and lipopolysaccharide-induced macrophages. *Biochem. Biophys. Res. Commun.* 1995, *214*, 839-846.