Evaluation of Pharmacological Properties of *Danshen* Compounds by Computational and Biochemical Approaches

A thesis submitted in fulfilment of the requirement for the degree of
Doctor of Philosophy

Zhi Wei Zhou
BSc Biotechnology & Biopharmacy

School of Health Sciences
Health Innovations Research Institute (HIRi)
RMIT University, Victoria, Australia
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Declarations

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to quality for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Zhi Wei Zhou

Date 18/12/2012
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Publications

Refereed Papers


Papers Submitted


Papers in Preparation


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Summary of Thesis

The metabolic and cardiovascular diseases are major health problems in many countries with significant health burden to the society. It has been recognized that endothelial dysfunction, caused by imbalance of responses to endothelium derived vasodilators and contracting factors, plays an important role in the pathogenesis of these diseases. Endothelial nitric oxide synthase (eNOS) uncoupling, occurred under oxidative stress due to excessive generation of reactive oxygen species, is a key driver to endothelial dysfunction. Functional eNOS requires adequate cofactors. Alterations of bioavailability of these cofactors in particular tetrahydrobiopterin (BH4) or other regulatory mechanisms such as heat shock protein (HSP) 90 can result in eNOS uncoupling. Identification of new agents to target eNOS uncoupling mechanism is an important direction for developing new therapies for treating endothelium dysfunction and related metabolic and cardiovascular diseases.

Danshen is the dried root of Salvia miltiorrhiza Bunge. It has long been used in traditional medicine for the treatment of cardiovascular and cerebrovascular diseases. The active components of Danshen, such as tanshinones, have certain actions on the endothelium. However, their actions on eNOS uncoupling have not been investigated. In addition, the relationship between chemical characteristics of Danshen compounds and their potential drug capability and the profile of absorption, distribution, metabolism, extraction and toxicity (ADME/Tox) are not fully understood. Thus, this project aims to evaluate the effects of Danshen compounds on eNOS uncoupling and the ADME/Tox profiles of natural compounds/products.

Firstly, the drug potential and toxicity risk of Danshen compounds were studied by using various established computational tools. Among 94 compounds evaluated, 76 compounds showed drug-like properties including major tanshinones-tanshinone I (Tan I), tanshinone IIA (Tan IIA) and cryptotanshinone (CT). Irritating effect is a potential toxicity risk of these compounds. Literature analysis indicates a number of commonly used natural compounds including Danshen compounds are the substrate or ligand of drug metabolizing enzymes, transporters, and nuclear receptors both in experimental and computational studies.
Secondly, a biochemical approach was used to study eNOS uncoupling in human umbilical vein endothelial cell line (EA.hy926), by using a high glucose induced eNOS uncoupling model. The EA.hy926 cells were incubated with 35 mM D-glucose for 24 hr or 48 hr. The cells were exhibited reduced nitric oxide (NO) and increased eNOS derived superoxide, reduced ratio of dimer/monomer of eNOS and BH4 bioavailability, and increased nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) expression. Phosphoinositide 3-kinase (PI3K) signaling pathway was also enhanced and HSP90 expression was decreased.

Using this cell model, three major tanshinones (Tan I, Tan IIA and CT) were then tested for their effects on eNOS uncoupling in EA.hy926. All three compounds significantly restored high glucose induced eNOS uncoupling, with increased BH4 bioavailability and NO production, reduced superoxide generation and the expression of NOX4, the major source of reactive oxygen species in endothelium cells, up-regulated the expressions of guanosine triphosphate cyclohydrolase I (GTPCH1), dihydrofolate reductase (DHFR) and HSP90, down-regulated the expression of PI3K. In addition, two synthetic analogues of tanshinones were also found with similar effects on eNOS uncoupling.

The findings from these studies suggest that computational analysis of Danshen compounds is capable of predicting their drug potential and toxicity risk. The study has also demonstrated for the first time the mechanism of actions of tanshinones and two synthetic compounds on eNOS uncoupling via regulation of BH4 bioavailability and the expressions of GTPCH1 and DHFR, reduction of the expression of NOX4 and up-regulation of HSP90. The regulation of NOX4 by tanshinones may be related to PI3K pathway. These findings indicate that tanshinones may be used as a prototype to develop new agents to treat eNOS uncoupling-mediated cardiovascular and metabolic diseases. Finally, an integration of computational approaches with well-established biochemical approaches make an important contribution in natural products research and drug discovery and development in the future.
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Abbreviations

•OH Hydroxyl
1\(^{1}\)O\(_{2}\) Singlet oxygen
3'-UTR 3'-mRNA untranslated region
3D/4D-QSAR Three/four dimension quantitative structure activity relationship
5-MTHF 5-Methyltetrahydrofolate
5'-UTR 5'-mRNA untranslated region
ADME/Tox Absorption, distribution, metabolism, extraction and toxicity
AGE Advanced glycation end products
ADMA Asymmetric dimethylarginine
Ang II Angiotensin II
AP-1 Activator protein 1
ApoE\(^{-/-}\) Apolipoprotein E-knockout
ATF Activating transcription factor 1
ATP Adenosine-5'-triphosphate
BH4 Tetrahydrobiopterin
BID BH3 interacting domain
BLT2 Leukotriene B4 receptor
CaM Calmodulin
CAR Constitutive androstane receptor
CEC Circulating endothelial cell
CoMFA Comparative molecular field analysis
COX Cyclooxygenase
CREB CRE-binding protein
CT Cryptotanshinone
CYP Cytochrome P450
DHFR Dihydrofolate reductase
DMF Dimethylformamide
DPI Diphenylene iodonium
DPPH 1,1-Diphenyl-2-picrylhydrazyl
E2F1 E2F transcription factor 1
EC Endothelial cell
EDHF Endothelium derived hyperpolarizing factor
EGF Epidermal growth factor
Elf-1 E74-like factor 1
eNOS Endothelial nitric oxide synthase
ERK Extracellular-regulated kinase
ET-1 Endothelin-1
FA Folic acid
FAD Flavin adenine dinucleotide
FAV Forearm volume
FDA Food and drug administration
FMN Flavin mononucleotide
GSH Glutathione

XVII
GSH-Px  Glutathione-peroxidase  
GTPCH1  Guanosine triphosphate cyclohydrolase I  
H$_2$O$_2$  Hydrogen peroxide  
HDL  High density lipoprotein  
HIF-1α  Hypoxia-inducible factor 1alpha  
HMG-CoA  3-Hydroxy-3-methyl-glutaryl-CoA reductase  
HNF-1A  Hepatocyte nuclear factor 1 homeobox A  
HOCl  Hypochlorous acid  
HoxA9  Homeobox A9  
HSP90  Heat shock protein 90  
HUVEC  Human umbilical vein endothelial cell  
ICAM-1  Intercellular adhesion molecule-1  
IL  Interleukin  
IFN-γ  Interferon-gamma  
iNOS  Inducible nitric oxide synthase  
IRE-1  Inositol-requiring enzyme 1  
IRF  Interferon regulatory factor  
JNK  c-Jun NH2-terminal kinase  
LDL  Low density lipoprotein  
L-NAME  $N^G$-nitro-L-arginine methyl ester  
LPS  Lipopolysaccharide  
MAPK  Mitogen activated protein kinase  
MCAO  Middle cerebral artery occlusion  
MDA  Malondialdehyde  
MEF2B  MADS box transcription enhancer factor 2, polypeptide B  
MI  Myocardial infarction  
NADH  Nicotinamide adenine dinucleotide  
NADPH  Nicotinamide adenine dinucleotide phosphate  
NF-1  Neurofibromatosis-1  
NF-κB  Nuclear factor-kappaB  
nNOS  Neuronal nitric oxide synthase  
NO  Nitric oxide  
NOS  Nitric oxide synthase  
NOX  NADPH oxidase  
O$_2^-$  Superoxide  
O$_3$  Ozone  
OATP  Organic anion-transporting polypeptide  
OCT  Organic cation transporter  
ONOO$^-$  Peroxynitrite  
PAF  Platelet-activating factor  
PASMC  Pulmonary artery smooth muscle cells  
PD  Pharmacodynamics  
PDGF  Platelet derived growth factor  
PEPT  Proton-coupled small peptide carrier
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2-alpha</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLSD</td>
<td>Partial least squares discriminant</td>
</tr>
<tr>
<td>Polidp 2</td>
<td>Polymerase delta-interacting protein 2</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PSA</td>
<td>Polar surface area</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>QM</td>
<td>Quantum mechanical</td>
</tr>
<tr>
<td>RO•</td>
<td>Alkoxyl</td>
</tr>
<tr>
<td>RO₂•</td>
<td>Peroxyl</td>
</tr>
<tr>
<td>Ro5</td>
<td>Rule-of-Five</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>STS</td>
<td>Sodium tanshinone IIA sulfonate</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulphotransferase</td>
</tr>
<tr>
<td>Tan I</td>
<td>Tanshinone I</td>
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<tr>
<td>Tan IIA</td>
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<tr>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Thr</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate glucuronosyltransferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
<tr>
<td>XOR</td>
<td>Xanthine oxidoreductase</td>
</tr>
<tr>
<td>ZnS4</td>
<td>Zinc tetra-coordinated cluster</td>
</tr>
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Chapter 1. General Introduction

1.1 Oxidative stress

Oxidative stress plays a causal role in the development of many important cardiovascular and metabolic diseases (Figure 1.1) (1, 2). It is a hallmark of the cardiovascular risk states, such as atherosclerosis and diabetes (3). Oxidative stress is caused by an increase in the generation of pro-oxidant molecules and/or a decrease in the antioxidants in a given cellular compartment, in which the pro-oxidants outweigh the capability of antioxidant systems, resulting in an imbalance in the amounts of oxidants and antioxidants.

![Figure 1.1. Oxidative stress and diseases.](image-url)
Under physiological conditions, cells produce certain amount of reactive oxygen species (ROS) such as superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) in a deliberately and tightly regulated manner to be used as second messengers in redox signalling pathways. However, under pathological conditions, the production of ROS in vascular cells is elevated. When these molecules are present at high concentrations and escape detoxification by cellular antioxidant pathways, they may undergo direct chemical interactions with other biomolecules. Of particular importance are the reactions between $O_2^-$ and NO, which gives rise to peroxynitrite (ONOO$^-$), and the iron-catalysed Haber-Weiss reaction between $O_2^-$ and $H_2O_2$, which gives rise to hydroxyl radicals ($•OH$). ONOO$^-$ and $•OH$ are extremely powerful oxidising species. Along with $O_2^-$ and $H_2O_2$, they can cause endothelial dysfunction through direct oxidative damage to cellular macromolecules, impairment of the NO signalling pathway, and activation of pro-inflammatory signalling cascades. Consequently, the normal redox state of the biological compartment is shifted towards oxidising states, which may potentially lead to oxidative damage to macromolecules such as proteins, lipids, nucleotides and carbohydrates.

Recently, accumulating evidence suggests that ROS production in vascular pathophysiology is a result of a complex feed-forward mechanism involving nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), a primary source of ROS leads to dysfunction of endothelial nitric oxide synthase (eNOS), xanthine oxidoreductase (XOR), cytochrome P450 system (CYP450) and the mitochondrial electron transport chain.

### 1.1.1 The sources of ROS

ROS comprises of many $O_2$ derived small molecules, including oxygen radicals [$O_2^-$, $•OH$, peroxy ($RO_2•$), and alkoxyl ($RO•$)] and certain nonradicals that are either oxidizing agents and/or are easily converted into radicals, such as hypochlorous acid (HOCl), ozone ($O_3$), singlet oxygen ($^{1}O_2$), and $H_2O_2$ (4). ROS can be generated from various sources (5). They can be produced under physiological conditions as intermediates in the cyclooxygenase (COX) or CYP450 (6); or as by-products of normal metabolism in the mitochondria (7). Under pathological condition, ROS generation is boosted through NOXs, XOR or NOS due to accidental electron
transfer onto $O_2$ during substrates or cofactors shortage (8, 9). Among these, NOXs are the major ROS generating enzyme family present in plants, yeast, and animals (10).

1.1.1.1 NOXs

NOXs are multicomponent enzymes mediating the transfer of electrons from cytosolic NADPH to $O_2$ to produce $O_2^-$ (11-13); it is a primary enzymatic source of ROS (4). NOXs are differentially expressed in the cardiovascular system and subject to regulation by various factors. Moreover, NOXs derived ROS are also important signalling molecules under physiological conditions. NOXs derived ROS serve as substrates for a variety of peroxidases such as myeloperoxidase, lactoperoxidase (14), and thyroid peroxidase (15). They also modulate signal transduction and gene expression (16). In addition, $O_2^-$, produced by NOXs, directly reacts with NO to form the highly oxidizing agent ONOO$^-$ (17). Accumulating evidence demonstrates that the endothelial NOX isoforms, including NOX1, 2, 4, 5, are the major contributors of ROS and they have been involved in the development of many cardiovascular diseases (18, 19). In addition, a cause-and-effect relationship among NOXs activity, vascular oxidative stress and diseases has been demonstrated using genetically modified mice that are deficient in either NOX1 (20-22) or NOX2 (23-26), or in the NOX organiser subunit p47phox (27). Thus, collectively, the above findings indicate that NOXs serve as a primary source of ROS generation in the vessel wall in various disease states. More detail about the NOX family will be discussed in Section 1.3.

1.1.1.2 XOR

XOR is a ubiquitous homodimeric enzyme playing a critical role in purine degradation (28, 29). XOR consists of two inter-convertible but functionally distinct enzymes, namely xanthine dehydrogenase (XDH), which is constitutively expressed, and xanthine oxidase (XO), which is generated by the posttranslational modification of XDH (30). Functionally, XORs act as a rate-limiting enzyme for the oxidation of hypoxanthine into xanthine and further into urate (30). For these reactions, XDH requires NAD$^+$ and XO instead of $O_2$ as an electron acceptor, thereby producing
nicotinamide adenine dinucleotide (NADH), $O_2^-$ and $H_2O_2$, respectively (30). Of note, under many conditions such as hypoxia, ischemia, or in the presence of various pro-inflammatory mediators, XD converts to XO either through reversible thiol oxidation of sulfhydryl residues on XD or via irreversible proteolytic cleavage of a segment of XD (30), which suggests that reversible thiol oxidation of sulfhydryl residues is a mechanism whereby XO activity may increase further oxidative stress (31).

XORs are expressed in high levels in the gut and liver, but low levels in the blood vessel wall. The regulation of expression and activity on both the transcriptional and posttranslational level has been described (32). For example, pro-inflammatory stimuli and hypoxia can significantly up-regulate the transcription of XO (32). It has been found that patients with coronary artery disease had a significant increase in XO activity in coronary arteries with high $O_2^-$ production, compared to control patients (33, 34). Furthermore, a variety of diseases such as coronary artery disease, heart failure, and hypertension have been shown with elevated XO levels. Interestingly, it has been demonstrated that there was a circulating form of XO, which can bind to glycosaminoglycans on the surface of ECs (35), and it has been shown that the activity of this endothelial bound XO dramatically increased by more than 200% in patients with chronic heart failure (36). Therefore, XO is most likely secondary to NOX as a source of ROS production in vascular pathophysiology.

1.1.1.3 Mitochondrial respiratory chain

Mitochondria is an important cellular organelle where the conversion of the oxidoreduction energy of mitochondrial electron transport to high energy phosphate bond of adenosine-5'-triphosphate (ATP) takes place through a multicomponent NADH dehydrogenase (37). However, during abnormal mitochondrial respiration, there is a leakage of electrons to molecular $O_2$ at complexes I (NADH dehydrogenase), II (succinate dehydrogenase) and III (ubiquinone-cytochrome bc1) of the electron-transport chain resulting in the generation of $O_2^-$ towards the mitochondrial matrix. As such, the mitochondrial respiratory chain can be a major source of $O_2^-$. 
An increasing body of evidence suggests that increased mitochondrial ROS generation is involved in the development of many cardiovascular and metabolic diseases (38). For example, a number of stimuli that are able to induce endothelial dysfunction have been shown to increase mitochondrial ROS production, such as adipokine, leptin, CD40, hypoxia, cyclic strain, oxidised low density lipoprotein (LDL), electrophilic lipid oxidation products and high glucose (39). Of importance, it has been demonstrated that excessive ROS generation by the mitochondria directly contributes to endothelial dysfunction and vessel diseases (40). Ohashi et al. (41) reported a reduced NO bioavailability and accelerated atherosclerotic lesion development in a ApoE\(^{-/-}\) mice model with genetic deletion of manganese SOD, whereas, Zhang et al. (42) showed a protective action on endothelial function against atherosclerosis in the same strain of mice by endothelial specific overexpression of the mitochondrial thioredoxin 2 gene. Moreover, it has been shown that mitochondrial derived O\(_2^-\) was increased in hypxia-reoxygenation and ischemia-reperfusion (43). There are also potential interactions between difference sources of ROS. For example, H\(_2\)O\(_2\) and ONOO\(^-\) may directly increase mitochondrial ROS production (44-46), and Doughan et al. (47) demonstrated excessive ROS generation by mitochondria may occur in the presence of elevated NOXs activity.

1.1.1.4 CYP450

CYP450 is one of the most important drug metabolizing enzymes responsible for Phase I drug metabolism. It is capable of converting exogenous and endogenous substances. The extra-hepatic expression of CYPs has been reported, such as in the cardiovasculature (48). CYP2 and CYP4A families are able to metabolize arachidonic acid which has been implicated in vascular regulation through the generation of vasodilators and vasoconstrictors (49-51).

There is evidence that vascular CYP enzymes can generate O\(_2^-\), H\(_2\)O\(_2\), and •OH during the CYP reaction cycle, when the electron flow is disturbed (49). The electron is transferred to the O\(_2\) instead of the central heme iron for reduction (49). It has been demonstrated that CYP2C is a significant source of ROS in cultured and native ECs and is involved in the endothelium derived hyperpolarizing factor (EDHF), a
vasodilator, response in porcine coronary arteries (52). Due to the capability of generating EDHF and ROS, it suggests that the outcome of altered CYP2 activity may be quite complex.

Endothelial CYP activity and expression can be regulated by cyclic stretch, hormonal stimuli, and 3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) (53), leading to both increased ROS and increased EDHF. Under pathological conditions such as hypertension and hypercholesterolemia, CYP expression has been reported to be increased (54). In the case of statins, an increase in eNOS expression may result in increased ONOO\(^-\) formation. On the other hand, oxidized LDL reduces endothelial CYP2 family enzyme expressions via ROS, which is probably generated by NOX and acting through reduced expression of the transcriptional regulator neurofibromatosis-1 (NF-1) (55).

1.1.1.5 eNOS

eNOS is one of the most important athero-protective enzymes in the blood vessel wall. It catalyses the formation of endothelium derived NO, which plays a critical role in the maintenance of a healthy vasculature homeostasis via its vasorelaxant, anti-inflammatory and anti-clotting actions (more details will be discussed in Section 1.4). However, under certain circumstances, eNOS may also act as a significant source of intracellular ROS (see Section 1.4), and contribute to vascular oxidative stress and endothelial dysfunctions in many cardiovascular and metabolic diseases (56-58).

1.1.2 Pathophysiological role of ROS

The pathophysiological function of ROS has been extensively investigated in the last decade (4, 59). It is known that ROS is involved in a wide range of cellular processes, implicated in a wide variety of pathophysiological conditions (4, 60-62), including host defence, inflammation, cellular signalling, gene expression, cellular death, cellular senescence, regulation of cell death, O\(_2\) sensing, biosynthesis, protein cross linking, regulation of cellular redox potential, reduction of metal ions, regulation of matrix metalloproteinases, angiogenesis, and cross link with the NO system.
Of particular importance, the role of ROS in endothelial function and vascular homeostasis has been reported (4). Firstly, ROS act as an oxygen sensing (63, 64). It has been reported that many types of acute and chronic O₂-sensitive processes were involved in cardiorespiratory homeostasis and O₂ sensitive alterations in endothelial function were essential for vascular homeostasis. For example, in the coronary circulation, a slight decrease in arterial O₂ pressure would result in a rapid increase in endothelial production of NO and vasodilator prostanoids, which directly increase blood flow and thus O₂ supply (65). Secondly, ROS can regulate vascular tone through interacting with NO and closely dependant on direct effects of H₂O₂ (4, 54).

It is well established that endothelium derived NO underwent a very rapid reaction with O₂⁻ that led to inactivation of NO. On the other hand, a number of studies indicate that H₂O₂ was involved in flow induced dilation (66, 67), which suggest that H₂O₂ production contributed to the physiological regulation of vascular tone in certain vascular beds. In addition, ROS also have the capability of regulating EC growth, migration, proliferation, and survival, in which the physiological functions have been demonstrated (4).

The pathological mechanisms of ROS are largely due to its capability of interacting with various molecules, such as proteins, lipids, carbohydrates and other small inorganic molecules to irreversibly destroy or alter the function of the target molecule. As a consequence, disturbed cellular processes such as uncontrolled cellular proliferation and apoptosis may occur in vasculature, which in turn results in endothelial dysfunction. It is suggested that all the alterations are crucial contributors to cardiovascular disease.

Consequently, ROS have been increasingly identified as major contributors to numerous disorders or diseases such as hypertension, aortic aneurysm, myocardial infarction, ischemia-reperfusion disorders, pulmonary fibrosis, amyotropic lateral sclerosis, Alzheimer’s disease, Parkinson’s disease, ischemic stroke, diabetic nephropathy, and renal cell carcinoma (68-70). Moreover, under certain conditions, such as hyperglycaemia, hyperlipidaemia and hyperinsulinemia, the excessive ROS production can result in more complications and dysfunctions (71-74).


1.2 **Endothelial dysfunction**

Endothelial dysfunction is characterized by the shift of endothelium mediated vasodilation due to a reduction of the bioavailability of vasodilators and an increase in endothelium derived contracting factors (75, 76). It has been implicated in the pathogenesis of many cardiovascular and metabolic diseases (Figure 1), such as coronary artery disease, atherosclerosis, chronic heart failure, peripheral artery disease, hypertension, diabetes, obesity and chronic renal failure (56, 77, 78).

It is proposed that the severity of endothelial dysfunction can be used as an independent predictor of cardiovascular events, providing valuable prognostic information. It has been known that certain cardiovascular risk factors and therapeutic intervention, including statins and angiotensin converting enzyme inhibitors, may modify endothelial function. Endothelial function may be an attractive primary target in the effort to optimize individualized therapeutic strategies to reduce cardiovascular morbidity and mortality.

1.2.1 **Pathogenesis of endothelial dysfunction**

The key mechanism of endothelial dysfunction is the disrupted balance of vasodilating and vasocontracting factors. Consequently, endothelium derived NO production is decreased and ROS generation is increased resulting in a decline in the bioavailability of NO and excessive accumulation of ROS, which in turn cause oxidative stress (1, 78).

1.2.1.1 **ROS**

A number of studies have been shown the causal role of excessive ROS in endothelial dysfunction resulting in the genesis and progression of cardiovascular diseases (79, 80). In an animal model, it has been shown that increased ROS production induced endothelial dysfunction, but supplementation of antioxidants improved endothelium-dependent relaxation by regulation of NOX and SOD (81). In hypertensive patients, Taddei et al. (82) showed that increased production of ROS impaired endothelium-dependent relaxation. Further to this, Heitzer et al. (83) showed that the relationship between NO and ROS was correlated with the degree of
impairment of endothelium-dependent vasodilation and cardiovascular events. In addition, this relationship was observed in chronic renal failure as well. In animal models, it has been demonstrated that increased ROS production resulted in decreased NO bioavailability and endothelial dysfunction, whereas pre-treatment with antioxidant may result in an improvement (84, 85). In patients, Annuk et al. (86) showed that oxidative stress markers were also correlated with endothelial dysfunction. Cross et al (87) observed improvement on endothelial dysfunction by the administration of vitamin C. Moreover, in diabetic animal models, it also has been shown that increased production of ROS caused endothelial dysfunction (88, 89).

1.2.1.2  NO

NO is the most important vasodilating substance catalysed by NOS. It has been reported that reduced NO level is often accompanied by impaired endothelial function. There are many reasons for the reduction of NO bioavailability. The two basic reasons are decreased generation and increased degradation. The reduced generation may result from impaired activity of NOS or increased diminish by NO quencher(s) to decreased bioavailability of NO, eventually, leading to endothelial dysfunction. It is well known that ROS are capable of quenching NO resulting in the formation of ONOO$^-$ with cytotoxic effects on protein function and endothelial function via protein nitration (90, 91). Moreover, ONOO$^-$ is an important mediator of oxidation of LDL (90, 91) and is able to degrade eNOS cofactor tetrahydrobiopterin (BH4) causing eNOS uncoupling (92).

1.2.1.3  Asymmetric dimethylarginine

Asymmetric dimethylarginine (ADMA) is a relatively new and attractive mechanism resulting in a reduction of NO bioavailability (93). It is an endogenous competitive inhibitor of eNOS (94, 95). It has been demonstrated that ADMA uncoupled eNOS leading to oxidative stress in human umbilical vein endothelial cells (HUVECs) (93). The role of ADMA in endothelial dysfunction also has been studied in vivo. It has been demonstrated that plasma ADMA level was correlated with the inhibition of eNOS in ECs from patients with chronic renal disease (96, 97). Besides, it has been
shown that ADMA levels were inversely related to endothelium-dependent vasodilation in subjects with hypercholesterolemia, whereas infusion of L-arginine and competitor of ADMA can normalize endothelial function (98). It also has been suggested that accumulation of ADMA led to reduced effect on renal plasma flow and increased renovascular resistance and blood pressure (99). Moreover, it has been demonstrated that supplementation with L-arginine, BH4 and vitamins can alleviate ADMA induced endothelial dysfunction (93, 100, 101).

1.2.1.4 Angiotensin II

Angiotensin II (Ang II) is a key vasoactive compound and has been implicated in the pathogenesis of many cardiovascular diseases (25). It has been reported that Ang II stimulated the activity and expression of NOXs resulting in increased ROS production, which in turn led to eNOS uncoupling (102-106). Ang II induced endothelial dysfunction also has been observed in animal models (107-109). On the other hand, the beneficial effects on endothelial function by antagonising Ang II receptors have been demonstrated (110). Sarr et al. (111) showed that red wine polyphenol can prevent Ang II induced hypertension and endothelial dysfunction, and normalize vascular ROS production and NOX subunit expression in rats. Zhang et al. (112) also reported that simvastatin can ameliorate Ang II induced endothelial dysfunction through restoration of Rho-BH4-eNOS-NO pathway. In hypertensive patients, it also has been shown that interruption of the renin-angiotensin system with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers restored endothelial function (113-115).

1.2.1.5 Hyperhomocysteinemia

Hyperhomocysteinemia has been recognized as a non-traditional cardiovascular risk factor causing endothelial dysfunction in both animal and human studies (95, 116). It has been shown that hyperhomocysteinemia induced endothelial dysfunction in methylenetetrahydrofolate reductase-deficient mice (116) and normotensive patients (117). Conversely, reducing homocysteine level can ameliorate endothelial function in both animal and human studies. For instance, a reduction of homocysteine levels and improvement of endothelial function can be observed after folic acid (FA)
supplementation in children with chronic renal failure (118). In addition, it has been demonstrated that homocysteine may inhibit dimethylarginine dimethylaminohydrolase leading to ADMA accumulation (119). Consistently, it has been confirmed that hyperhomocysteinemia induced endothelial dysfunction is via accumulation of ADMA (95, 120). These findings may explain the increased cardiovascular risk of patients with hyperhomocysteinemia. This is of special importance for patients with chronic renal failure, who often have increased homocysteine levels (121).

1.2.1.6 Others

Other mechanisms may also be implicated in the pathogenesis of endothelial dysfunction. For example, under conditions of insulin resistance, insulin signalling pathway is altered with significant down-regulation of phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent kinase-1, and Akt/protein kinase B to phosphorylation and activation of eNOS, whereas the pathway leading to mitogenic effects and growth via mitogen activated protein kinase (MAPK) is unaffected (122-125).
1.3 NOX Family

NOX is a major superoxide generating enzyme family, which was firstly identified in phagocytic cells (126). The function of NOX was observed before the identification. It was responsible for the respiratory burst (127), and essential to the microbicidal function of these cells (11, 12, 128-131). NOXs transfer electrons from the cytosolic donor, NADPH, to flavin adenine dinucleotide (FAD), then sequentially to each of the two heme groups and then to O₂ on the opposite side of the membrane to generate ROS (132). Although all NOXs use this mechanism to generate ROS, modes of activation, subunit requirements, and subcellular localizations differ between isoforms (Table 1.1).
<table>
<thead>
<tr>
<th>Isoform</th>
<th>Human gene locus</th>
<th>Coupling components</th>
<th>Major distribution sites</th>
<th>Induction</th>
<th>Signalling molecules</th>
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<tbody>
<tr>
<td>NOX1</td>
<td>Xq22</td>
<td>p22phox NOXA1/p67phox NOXO1/p47phox p22phox NOXA1/p67phox NOXO1/p47phox</td>
<td>colon epithelium VSMC</td>
<td>IFN-γ, flagellin, IL-1β, TNF-α (CCE); Ang II, PDGF, PGF2α, TPA, serum, aldosterone/high salt (VSMC); LPS (macrophage, gastric mucosal cell); IL-1α (mast cell)</td>
<td>GATA-6, HNF-1A, Cdx1 (constitutive expression in CEC); STAT1 (induction in CEC); PKCδ, EGF receptor, PI3K, ATF-1, MEF2B, ERK1/2, JunB (induction in VSMC); NF-κB (induction by LPS); LTB4-BLT2 receptor (induction by IL-1β in mast cell)</td>
</tr>
<tr>
<td>NOX2</td>
<td>Xp21.1</td>
<td>p22phox p67phox p47phox p40phox Rac</td>
<td>phagocyte</td>
<td>IFN-γ, LPS (phagocyte); TPA, retinoic acid/DMF (myeloid cell)</td>
<td>BID, IRF-1, IRF-2, CP1, Elf-1, PU.1 (phagocyte); HoxA9 (myeloid differentiation); GATA-1 (eosinophil); NF-κB (induction by LPS)</td>
</tr>
<tr>
<td>NOX3</td>
<td>6q25.3</td>
<td>p22phox NOXA1/p67phox NOXO1/p47phox Rac</td>
<td>inner ear</td>
<td>hypoxia (PASMC); TGF-β (cardiac fibroblast, SMC); 7-ketocholesterol (VSMC); cannabidiol (leukemia cell)</td>
<td>HIF-1α (induction by hypoxia); Smad3 (induction by TGF-β); IRE-1, JNK, AP-1 (induction by 7-ketocholesterol); E2F1 (constitutive expression in VSMC)</td>
</tr>
<tr>
<td>NOX4</td>
<td>11q14.2-q21</td>
<td>p22phox</td>
<td>kidney (many other organs)</td>
<td>hypoxia (PASMC); TGF-β (cardiac fibroblast, SMC); 7-ketocholesterol (VSMC); cannabidiol (leukemia cell)</td>
<td></td>
</tr>
<tr>
<td>NOX5</td>
<td>15q22.31</td>
<td></td>
<td>spleen, testis, lymph node</td>
<td>PAF (Barrett’s esophageal adenocarcinoma)</td>
<td>STAT5 (induction by PAF)</td>
</tr>
<tr>
<td>DUOX1</td>
<td>15q21</td>
<td>DUOXA1</td>
<td>thyroid</td>
<td>IL-4, IL-13 (airway epithelial cell)</td>
<td></td>
</tr>
<tr>
<td>DUOX2</td>
<td>15q15.3</td>
<td>DUOXA2</td>
<td>thyroid</td>
<td>IFN-γ (airway epithelial cell)</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Katsuyama et al. (60, 133).
Abbreviation: Ang II, angiotensin II; AP-1, activator protein 1; ATF, Activating transcription factor; BID, BH3 interacting domain; BLT2, leukotriene B4 receptor; CEC, circulating endothelial cells; DMF, dimethylformamide; E2F1, E2F transcription factor 1; EGF, epidermal growth factor; Elf-1, E74-like factor 1; ERK, extracellular signal regulated kinases; HIF-1α, hypoxia-inducible factor 1alpha; HNF-1A, hepatocyte nuclear factor 1 homeobox A; HoxA9, homeobox A9; IFN-γ, interferon-gamma; IL-1α, interleukin-1alpha; IL-1β, interleukin-1beta; IRE-1, inositol-requiring enzyme 1; IRF, interferon regulatory factor; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MEF2B, MADS box transcription enhancer factor 2, polypeptide B; NF-κB, nuclear factor-kappaB; NOX, NADPH oxidase; PAF, platelet-activating factor; PASMC, pulmonary artery smooth muscle cell; PDGF, platelet derived growth factor; PGF2α, prostaglandin F2-alpha; PI3K, phosphoinositide 3-kinase; PKCδ, protein kinase C delta; SMC, smooth muscle cell; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-alpha; TPA, 12-O-tetradecanoylphorbol-13-acetate; VSMC, vascular smooth muscle cell.
Currently, the differential expression of various isoforms has been described in mammalian species (4), and four of these isoforms were observed in vasculature. They are NOX1 expressed in ECs and VSMCs, NOX2 expressed in ECs, adventitial fibroblasts, neutrophils, macrophages, T cells and platelets, NOX4 expressed in ECs, VSMCs and adventitial fibroblasts, and NOX5 expressed in ECs.

1.3.1 NOXs structure
To date, seven members of the NOX family have been distinguished on the basis of the membrane spanning catalytic subunits of NOX or DUOX which they utilise to transfer electrons from NADPH to O$_2$ and produce ROS (Figure 1.2). They are NOX1, 2, 3, 4, 5 as well as DUOX1- and 2-containing oxidases (4, 134-137). The core structure of all NOX isoforms consists of six conserved transmembrane domains and a cytosolic C-terminal. Some conservative structural properties of NOX isoforms are common to all family members, including an NADPH-binding site and a FAD-binding region at C-terminal, four highly conserved heme-binding histidines. In DUOX proteins, the histidines are in the fourth and sixth transmembrane domains. Additional features, such as EF hands, an additional N-terminal transmembrane domain, and/or a peroxidase homology domain, are limited to DUOX isoforms (Figure 1.2).
Figure 1.2. Seven mammalian NOX isoforms.

The catalytic core subunits of the enzymes (NOX1-NOX5, DUOX1 and DUOX2) are shown in pink. All the required subunits and regulatory protein are labelled. EF hand motifs are also shown, which bind to Ca$^{2+}$ and thereby regulate the activity of NOX5, DUOX1 and DUOX2 oxidases. The figure also illustrates the putative additional amino-terminal transmembrane domain and extracellular peroxidase-like region on DUOX1 and DUOX2. Although NOX3 oxidase activity is enhanced by the expression of organizer and activator proteins, the enzyme displays constitutive activity in their absence.
It has been demonstrated that NOXs may require up to five additional protein subunits for their function, including a small membrane-bound protein (p22phox), which forms a heterodimer with certain NOX proteins to stabilise their expression within biological membranes; cytosolic ‘organiser’ (p47phox or Noxo1) and activator (p67phox or Noxa1) proteins; a third cytosolic protein (p40phox) that is known to associate with the NOX2-containing enzyme complex but whose function remains to be defined; and a small GTPase (Rac1 or 2) (4, 135, 138-140). Additionally, the activity of each NOX is associated with regulatory proteins with the NOX catalytic subunits in variable manners (Table 1.1). For example, NOX1 and NOX2 are constitutively associated with p22phox; and the full activity of NOX1/p22phox requires several cytosolic proteins including Rac, NOXO1 (or its homolog, p47phox) and NOXA1 (or its homolog, p67phox); Rac, p40phox, p47phox, and p67phox are required for activation of NOX2/p22phox. Conversely, there is no requirement of additional cytosolic proteins for NOX4 and NOX5 activation. NOX4 is constitutively associated with p22phox (141); whereas NOX5 is activated in a Ca$^{2+}$-dependent manner (142, 143).

1.3.2 NOX isoforms

1.3.2.1 NOX1

Human NOX1 gene is located on the X chromosome. The molecular mass of NOX1 is in the range of 55-60 kDa and it is the first homology of NOX2 reported (144-146). There are several binding elements in the 5’-region of the human NOX1 gene, including signal transducers and activators of transcription (STATs), interferon regulatory factor (IRF), activator protein 1 (AP-1), nuclear factor-kappaB (NF-κB), CRE-binding protein (CREB), CREB binding protein/p300 elements (147), and GATA factors (148). It is suggested that NOX1 can be induced under certain circumstance (147). In addition, Functional NOX1 requires cytosolic and membrane subunits to reduce O$_2$ and produce O$_2^-$ (4, 144, 149).

The expression of NOX1 was found in a variety of cell types and tissues, including VSMCs (150, 151), ECs (152, 153), uterus (151, 154), placenta (146), prostate (151, 154), osteoclasts (155), and retinal pericytes (156). In addition, NOX1 was also
expressed in several cell lines, such as the colon tumor cell line Caco-2 (157, 158), DLD-1 (157), HT-29 (157) and the pulmonary epithelial cell line A549 (159). Above all, NOX1 was the most highly expressed in colon epithelium (151, 160, 161).

In addition to the constitutive expression, NOX1 expression can be induced by PDGF, prostaglandin F2α, and Ang II in VSMCs (150, 151, 162, 163). An increased expression of NOX1 was also observed in carotid artery after injury (164) and in prostate in response to castration (165).

Since the expression of NOX1 was found in vasculature (150, 151), increasing interest has been attracted on the role of NOX1 in vascular function. It has been demonstrated that NOX1 played a role in the pathogenesis of many cardiovascular diseases (4, 20, 166-169). Recently, Youn et al. (170) showed that activation of NOX1 caused eNOS uncoupling in a streptozotocin induced murine model of diabetes. Sheehan et al. (167) demonstrated that NOX1 was implicated in the development of atherosclerosis by using apolipoprotein E-knockout (ApoE<sup>-/-</sup>) and NOX2-knockout/apolipoprotein E-knockout (ApoE<sup>-/-</sup>/NOX1<sup>-/-</sup>) mice. Lee et al. (166) showed that increased expression and activation of NOX1 in VSMCs led to increased degradation of extracellular matrix, cellular migration, and proliferation. Additionally, in a murine model of hypertension, the overexpression of NOX1 in VSMCs resulted in increased blood pressure and aortic hypertrophy compared to control mice receiving Ang II (20, 168). Moreover, NOX1 deficient mice were protected from Ang II induced hypertension and exhibited increased NO bioavailability, reduced aortic VSMCs hypertrophy, and decreased extracellular matrix deposition (21, 171). These studies indicate that NOX1 may act a potential therapeutic target for the treatment of cardiovascular diseases.

1.3.2.2 NOX2

NOX2, also known as gp91phox, is the first NOX isoform discovered in neutrophils and macrophages and it was highly expressed in leukocytes (4). Its C- and N-terminals face towards the cytoplasm (4). Human NOX2 gene is located on the X chromosome and the expression of NOX2 gene can be regulated by a number of
repressing and activating factors (4). Human NOX2 is a highly glycosylated protein (172, 173). NOX2 has the widest distribution among the NOX isoforms, including thymus, small intestine, colon, spleen, pancreas, ovary, placenta, prostate, and testis (174). The wide distribution is partly due to the presence of phagocytes. Moreover, NOX2 is also expressed in many other nonphagocytic cells, including neurons (175), cardiomyocytes (176), skeletal muscle myocytes (177), hepatocytes (178), ECs (17, 179, 180), and hematopoietic stem cells (181).

Like NOX1, it also has been demonstrated that NOX2 plays an important role in the pathogenesis of vascular diseases (4). Sorescu et al. (182) reported a positive correlation between the levels of the NOX2/p22phox mRNAs and the severity of atherosclerosis in atherosclerotic human coronary arteries. It also has been reported that active NOX2 is required for upregulation of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α); adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and intercellular adhesion molecule-1 (ICAM-1); and proinflammatory transcription factors, such as NF-κB. All these factors were involved in vascular inflammation and atherogenesis (183, 184).

Moreover, Judkins et al. (185) showed an increase in NO generation and a decrease in \( \text{O}_2^\cdot \) production and a reduction of neointimal formation and atherosclerotic lesion of the abdominal aorta in NOX2\(^{-/-}\)/ApoE\(^{-/-}\) model compared to single ApoE knockout model. Jung et al. (24) reported that NOX2 deficiency abolished Ang II induced increase in blood pressure and vascular hypertrophy, and protected endothelial function in a NOX2 deficient mice model. Bendall et al. (186) showed that NOX2 knockout mice were less vulnerable to Ang II induced cardiac hypertrophy and interstitial cardiac fibrosis. Looi et al. (187) demonstrated that NOX2 deletion in mice protected against post-myocardial infarction hypertrophy, fibrosis, and left ventricular dysfunction. Krijnen et al. (188) reported that NOX2 expression was higher in infarcted than noninfarcted myocardium from patients who have died from myocardial infarction.

Therefore, like NOX1, NOX2 appears to be an attractive therapeutic target for the treatment of cardiovascular disease. However, inhibition of NOX2 in leukocytes may
result in immune dysfunction. For example, NOX2 deficiency led to an X-linked chronic granulomatous disease, an immune disorder (189). So, although NOX2 seems to be a desirable therapeutic target, the potential side effect of immunodeficiency highlights the importance of therapeutics that selectively target pathological NOX2 activation in cardiovascular cell types while leaving physiologic NOX2 activation in neutrophils intact.

1.3.2.3 NOX4

Human NOX4 gene is located on chromosome 11 and NOX4 was originally identified in the kidney with ~39% identity to NOX2 (190, 191). NOX4 is a p22phox-dependent enzyme without requirement of cytosolic subunits (190-192) and is a constitutively active NOX isoform (4). It is highly expressed in the kidney. In addition, NOX4 is distributed in osteoclasts (193, 194), ECs (153, 195, 196), smooth muscle cells (145, 150, 197-200), hematopoietic stem cells (181), fibroblasts (201-203), keratinocytes (204), melanoma cells (205), and neurons (206).

The expression NOX4 is also subject to regulation under many circumstances. For example, the expression of NOX4 mRNA or protein can be induced under the conditions of endoplasmic reticulum stress (199), shear stress (207), carotid artery injury (164), hypoxia and ischemia (206, 208), and transforming growth factor beta1 (TGF-β1) and TNF-α stimulation of smooth muscle (200, 209), Ang II stimulation (163, 210, 211). Downregulation of NOX4 mRNA and protein was also observed in response to peroxisome proliferator-activated receptor (PPAR)-γ ligands (212).

Recently, the role of NOX4 in the development of cardiovascular diseases has been drawn a great deal of attention. Due to the constitutively active profile, the expression of NOX4 is more suspicious in the pathogenesis of cardiovascular diseases. NOX4 has multiple roles and sometimes plays contradictory roles in ECs, VSMCs, cardiomyocytes, and fibroblasts, suggesting that the role of NOX4 is inconclusive in the development of cardiovascular diseases (213). For example, NOX4 exerted a vicious effect in ECs by promoting the expression of endothelial surface adhesion molecules, adhesion of monocytes, and the transmigration of
leukocytes into the vasculature (214); in addition, NOX4 played a vicious role in promoting the migration, proliferation, and differentiation of both VSMCs and ECs (215, 216). However, recently, Ray et al. (217) showed a beneficial effect of NOX4 a transgenic model of NOX4 overexpression in ECs, such as NOX4-dependent vasodilation. Moreover, It has been shown that NOX4 overexpression in cardiomyocytes protected against contractile dysfunction and hypertrophy, whereas NOX4 deletion promoted these deleterious cardiac effects (218). Although the function of NOX4 in cardiovascular diseases remains incompletely understood, available evidence suggests that NOX4 may have a complex role in cardiovascular diseases development.

1.3.2.4 NOX5

NOX5 is the most recently characterized NOX isoform (143, 174). The human NOX5 gene is located on chromosome 15 (4). NOX5 is without glycosylation and the molecular weight is 85 kDa (219). The mRNA expression of NOX5 was observed in a variety of tissues, including testis, spleen, lymph nodes, vascular smooth muscle, bone marrow, pancreas, placenta, ovary, uterus, stomach, and in various fetal tissues (143, 174, 220). NOX5 functions without the requirement of p22phox, which has been demonstrated by siRNA suppression of p22phox (149). The results showed that p22phox suppression led to a decrease in the activity of NOX1 to NOX4, but not of NOX5 (149). In addition, NOX5 does not require cytosolic organizer or activator subunits (143). It has been shown that NOX5 functions without the requirements of any cytosolic proteins (221).

Since the expression of NOX5 was observed in ECs and VSMCs, the role of NOX5 in the development of cardiovascular diseases has been focused. Montezano et al. (222) showed that NOX5 was activated by Ang II and endothelin 1 (ET-1) in ECs. Overexpression of NOX5 could result in ECs proliferation and lead to angiogenesis (142). Moreover, Jay et al. (223) showed that silencing NOX5 can decrease PDGF induced ROS production and inhibit proliferation of VSMCs. Furthermore, expression of NOX5 mRNA and protein was increased in human coronary arteries with atherosclerosis (224). These findings suggest that NOX5 has a significant role
in the pathogenesis of cardiovascular diseases. Further studies are needed to elucidate its specific role in the development of vascular diseases and determine whether it may act as an appropriate therapeutic target.

The remaining three NOX isoforms are NOX3, DUOX1, and DUOX2, which are not known to be expressed in the human vasculature. In addition, to date, there is no correlation between these isoforms and the development of cardiovascular diseases (4). Therefore, these NOX isoforms are unlikely to have been implicated in the pathogenesis of cardiovascular diseases.

1.3.2.5 NOX3

NOX3 shares ~56% amino acid identity with NOX2. The human NOX3 gene is located on chromosome 6, and there is no splice variants of NOX3 reported (4). Although the expression of NOX3 cannot be found in human vasculature, the expression of NOX3 still has a wide distribution in a variety of tissues. A high level of NOX3 can be expressed in the inner ear, including the cochlear and vestibular sensory epithelia, and the spiral ganglion (225). Besides, to a lesser extent, NOX3 was expressed in fetal spleen (226), fetal kidney (174, 225), skull bone, and brain (225).

Like most of the NOX isoforms, subunits are required for functional NOX3. NOX3 is a p22phox-dependent enzyme for activation (227, 228). Truncated p22phox inhibited NOX3 mediated ROS generation (227). NOXO1 was a required subunit for NOX3 activation which constitutively activates NOX3 (225, 228-230). p47phox and p67phox also have the capability of activating NOX3 (225, 228-230). In addition, it has been reported that the role of NOXA1 and Rac in NOX3 function is contradictory (228, 229).

1.3.2.6 DUOX1 and DUOX2

DUOX1 and DUOX2 were initially identified as H₂O₂-generating enzymes in the thyroid gland in a Ca²⁺- and NADPH-dependent manner (231-233). Both human DUOX genes are located on chromosome 15 (234). They are arranged in a head-to-
head configuration but separated by a 16 kb region with the direction of transcription away from one another (234). The structure of DUOX consists of NOX-like region at the C-terminal, two EF-hands, a membrane-spanning region, and a peroxidase like domain at the N-terminal (235). DUOX enzymes are glycosylated (236).

Like the other NOX isoforms, DUOX isoforms are expressed in a variety of tissues. Both DUOX1 and DUOX2 are highly expressed in the thyroid (233, 237). In addition, DUOX1 has been described in airway epithelia (238-240) and in the prostate (241). DUOX2 is found in the ducts of the salivary gland (239); in rectal mucosa (239); all along the gastrointestinal tract including duodenum, colon, and cecum (242, 243); in airway epithelia ((238, 240); and in the prostate (241).

DUOX generated H$_2$O$_2$ essential for the synthesis of thyroid hormones by thyroid peroxidase (244-246). Functionally, unlike the other NOX isoforms, DUOX does not require the other components of NOX except for two DUOX maturation factors called DUOXA1 and DUOXA2 (247). DUOXA1 and DUOXA2 are necessary for the formation of DUOX1 and DUOX2 functional complexes, respectively by playing an essential role in ER-to-Golgi transition, maturation, and targeting to the plasma membrane of DUOX1 and DUOX2 (247).

Recently, differential regulation of DUOX enzymes has also been reported. Harper et al. (248) reported an induction of DUOX1 in response to interleukin- (IL) 4 and 13; whereas DUOX2 was induced by interferon-gamma (IFN-γ) in respiratory tract epithelium. Moreover, induction of DUOX2 was found in response to insulin in thyroid cell lines (235), and during spontaneous differentiation of postconfluent Caco-2 cells (243). Rigutto et al. (249) revealed a differential activation of DUOX1 and DUOX2 by protein kinases. DUOX1 activity was stimulated by forskolin via protein kinase A (PKA) by phosphorylation on Ser955, phosphorylation of DUOX2 was induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) via PKC activation (249). Differential regulation of DUOX1 and DUOX2 might be important for host defence and inflammation.
1.3.3 NOX subunits and regulatory protein

The NOX subunits and regulatory protein are essential for the superoxide generating function and activity of NOX isoforms. The subunits and regulatory proteins required for NOX activation include membrane bound and cytosolic proteins. p22phox, which is membrane bound protein helping stabilize the NOX proteins and dock cytosolic factors; p47phox, p67phox, the small GTPase Rac, and the modulatory p40phox, which are cytosolic proteins working together to activate the NOX enzymes (4, 60, 133). The requirement of other components for NOX isoforms varies. For example, NOX2 requires at least five extra components for its activation, including Rac, p40phox, p67phox, p47phox and p22phox; NOX5 activation is dependent on Ca^{2+}, it does not need other components for its activation; DUOX1 and 2 require DUOXA1 and DUOXA2, respectively.

1.3.3.1 p22phox

The human p22phox gene is located on chromosome 16 and p22phox is a membrane protein (250). p22phox is required for the activation of NOX1 (251, 252), NOX2 (253, 254), NOX3 (149, 228), and NOX4 (144, 192). The p22phox gene and protein are widely expressed in both fetal and adult tissues (174) and cell lines (254), including phagocyte (255), ECs (256), VSMCs (257), and transfected cells (144, 192). Induction of p22phox expression has been described. For instance, Ang II can induce the expression of p22phox (103). The expression of p22phox was increased under the conditions of streptozotocin induced diabetes (258), and hypertension (259).

Binding with NOX proteins to stabilize proteins and binding to organizer subunits are two major functions of p22phox. Of note, it is revealed that only heterodimer of NOX proteins and the p22phox protein are stable, while monomers are degraded by the proteasome (253). The role of p22phox in interaction with organizer subunits is referred to NOX1, NOX2, and NOX3, but not to NOX4. It is believed that the proline-rich C-terminal of p22phox is responsible for the interaction with SH3 domains of the organizer subunits p47phox or NOXO1 (260, 261).
1.3.3.2 **p47phox and NOXO1**

p47phox (alias NOXO2) and NOXO1 are known as NOX organizers (262, 263). The human *p47phox* and *NOXO1* genes are located on chromosomes 7 and 16, respectively (264). NOXO1 and p47phox share ~25% sequence identity and a high degree of similarity in their functional domains (4, 60, 133). Both NOXO1 and p47phox have two SH3 domains that interact with p22phox in C-terminal proline-rich regions (252, 261). Besides, both NOXO1 and p47phox structures are also composed of a proline-rich region in C-terminal that enables NOXO1 and p47phox to interact with SH3 domains in NOXA1 and p67phox, respectively (252, 265). In addition, the structures of NOXO1 and p47phox contain phox domains that can interact with membrane phospholipids. Auto-inhibitory region is occurring in p47phox but not NOXO1 (4, 60, 133).

The expression of both NOXO1 and p47phox are detected in a variety of cells and tissues. p47phox is highly expressed in myeloid cells (266, 267), to a lesser extent, testis (264), inner ear (225, 264), lung (268), neurons (269), hepatocytes (178), hepatic stellate cells (270), glomerular mesangial cells (271), ECs (179), and VSMCs (272). The high expression level of NOXO1 is detected in the colon (160, 229, 252, 273), to a lesser extent, testis, small intestine, liver, kidney, pancreas, uterus, and inner ear (160, 252, 264). In addition, the regulation of p47phox and NOXO1 has been described. The expression of p47phox was induced under the conditions of retinoic acid induced differentiation of monocytes and granulocytes (266), streptozotocin induced diabetic rat kidney (274), and thrombin stimulated SMCs (272). It also has been demonstrated that *Helicobacter pylori* lipopolysaccharide (LPS) activated transcription of NOXO1 in guinea pig gastric mucosa (275).

1.3.3.3 **p67phox and NOXA1**

p67phox (alias NOXA2) and NOXA1 are known as activator subunits of NOX isoforms. The human *p67phox* and *NOXA1* genes are located on chromosomes 1 and 9, respectively. There is no splice variants of p67phox and NOXA1 reported so far (4, 60, 133). Both p67phox and NOXA1 are cytoplasmic proteins without glycosylation. The molecular weight of p67phox and NOXA1 is 67 and 51 kDa, respectively.
Despite of being only ~28% amino acid identity, both p67phox and NOXA1 have a high overall structural similarity. Both p67phox and NOXA1 have an N-terminal tetratricopeptide repeat, a highly conserved activation domain, a less conserved “Phox and Bem 1” domain, and a C-terminal SH3 domain (4, 60, 133).

Both p67phox and NOXA1 are expressed in a variety of tissues and cell types. p67phox was expressed in phagocytes (276), B lymphocytes (277), glomerular mesangial cells (271), ECs (179), neurons (278), astrocytes (278), kidney (279), and hepatic stellate cells (270). NOXA1 was expressed in the spleen, inner ear (225), stomach, colon, small intestine, uterus (225, 273), prostate, lung, thyroid, salivary glands (273), guinea pig gastric mucosal cells (275), basilar arterial epithelial cells (153), airway-like normal human bronchial epithelial cells (280), and VSMCs (281). It has been shown that the expression of p67phox can be modulated under certain circumstance. The expression of p67phox could be induced in response to a variety of stimuli, including IFN-γ in myelomonocytic U937 cells (282) and promyelocytic HL60 cells (283), zinc in neurons and astrocytes (278), and Ang II in aortic adventitial fibroblasts (284). It has been demonstrated that p67phox can also be phosphorylated (277, 285-291). NOXO1 constitutively associates with the membrane and so far it is unknown whether NOXA1 can be phosphorylated (292).

1.3.3.4  p40phox

p40phox is a nonglycosylated cytosolic protein with a molecular weight of 40 kDa (293). It was detected by coimmunoprecipitation with p47phox and p67phox (293). The human *p40phox* gene is located on chromosome 22. So far, there is a splice variant of p40phox identified (294). p40phox structure contains a SH3 domain, a phox domain, and a phox and bem1 domain. It has been shown that p40phox interacted with p47phox and p67phox with a 1:1:1 stoichiometry (295). The expression of p40phox can be detected in a variety of tissues and cell types, including phagocytes (293), B lymphocytes (277), spermatozoa (296), hippocampus (269), and vascular smooth muscle (102). It has been shown that Ang II is capable of inducing p40phox expression in rostral ventrolateral medulla (297) and vascular smooth muscle (102). It also has been reported that p40phox is involved in the
regulation of NOX2. Evidence shows that p40phox enhanced oxidase function (298-300). Of note, unlike p47phox and p67phox, it has been demonstrated that p40phox was indispensable for NOX2 activity (301), suggesting that p40phox is specific for NOX2.

### 1.3.3.5 Rac GTPases

Rac GTPases have a complex role in regulating cellular functions (302-306). There are three highly homologous Rac proteins in mammals, which are Rac1, ubiquitously distributed; Rac2, mostly expressed in myeloid cells; and Rac3, predominantly distributed in the central nervous system. It has been demonstrated that Rac proteins were involved in the NOX isoforms mediated $O_2^-$ generating process (4). Rac1 participated into the activation of NOX1 and NOX3 isoforms and Rac1 and Rac2 proteins were involved in the regulation of NOX2 (60). The activation of NOX4 appears to be Rac independent upon heterologous expression {Martyn, 2006 #2284, 133). Moreover, it has been demonstrated that NOX5 and DUOX-dependent ROS generation did not require Rac proteins activation (307).

### 1.3.4 Regulation of NOXs

Increased activity and expression of NOX isoforms has been demonstrated in a wide variety of diseases and/or disorders. In particular, all cardiovascular pathologies were marked by the excessive NOX generated ROS which were resulted from up-regulation of various NOX family members (64, 213, 308). In the past decade, extensive attention has been focused on the redox control and the underlying molecular mechanisms of NOX-dependent ROS generation. In addition, the regulation on NOX isoforms has also been studied (Table 1. 2). It has been shown that the regulation of NOX isoforms varies from transcriptional level to posttranslational level. Although the underlying mechanisms of NOX regulation have not been fully revealed, it has been reported that NOX1 and NOX2 can be induced by cytokines and mitogens, which may be implicated in the development of oxidative stress induced vasoconstriction, inflammation, and phenotypic alterations in the vessel wall. In addition, it has been shown that NOX4 can constitutively produce ROS and does not require regulatory subunits for the activation, unlike
NOX1 and NOX2. In this respect, it suggests that the regulation on NOX4 transcriptional level is more important. Collectively, numerous pro-inflammatory and growth-related factors can regulate NOX1, NOX2, NOX4, and NOX5 in vitro and in vivo (163, 209, 222, 309, 310).
Table 1. The activity, regulation, expression and function of the main NOXs in the cardiovascular system

<table>
<thead>
<tr>
<th></th>
<th>NOX1</th>
<th>NOX2</th>
<th>NOX4</th>
<th>NOX5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Constitutive activity</strong></td>
<td>Absent or very low</td>
<td>Absent or very low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Requirement for p22phox</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Essential regulatory subunits</strong></td>
<td>NOXO1, NOXA1, Rac</td>
<td>p67phox, p47phox, p40phox, Rac</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>Posttranslational modifications of regulatory subunits</td>
<td>Posttranslational modifications of regulatory subunits</td>
<td>Transcriptional. Can be regulated by Polidip2</td>
<td>Calcium binding</td>
</tr>
<tr>
<td><strong>Cell expression</strong></td>
<td>Vascular smooth muscle, possibly ECs</td>
<td>ECs, cardiomyocytes, fibroblasts, human vascular smooth muscle, inflammatory cells</td>
<td>ECs, cardiomyocytes, fibroblasts, vascular smooth muscle cells</td>
<td>Human endothelium, human vascular smooth muscle</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td>Constitutive low level superoxide production; VSMCs migration, proliferation; mitochondrial function</td>
<td>Phagocytic respiratory burst; endothelial function and NO signalling; neointimal formation</td>
<td>VSMCs growth and migration, angiogenesis; cardioprotective function? Constitutive source of H₂O₂?</td>
<td>ECs/VSMCs proliferation, cell migration; angiogenesis</td>
</tr>
</tbody>
</table>

Adapted from Touyz et al. (68) and Sirker et al. (311).
Abbreviation: EC, endothelial cell; H₂O₂, hydrogen peroxide; NOX, nicotinamide adenine dinucleotide phosphate oxidase; Polidp 2, polymerase delta-interacting protein; VSMCs, vascular smooth muscle cells.
1.3.4.1 Gene expression

The transcriptional regulation of NOX family members is essential to their function. It has been described that inflammation induced alterations on the gene expression of NOX isoforms are correlated with the increase in the production of ROS. Although the transcriptional mechanisms are not totally deciphered, it is suggested that multiple transcription factors were co-ordinately involved in the regulation of NOXs gene expression (60, 133).

It has been shown that AP-1 was essential for the rat NOX1 promoter activity (312, 313). Manea et al. (314) also reported that MAPK related pathways were involved in the modulation of NOX expression in human aortic smooth muscle cells. In addition, it was identified that the GATA binding site at -135/-130 on human NOX1 gene was essential for the basal transcriptional activity (148). In murine macrophages, it has been shown that the induction of NOX1 by LPS is partially mediated by C/EBPβ and C/EBPδ (315). Katsuyama et al. (316) demonstrated that activating transcription factor 1 (ATF-1), a transcription factor of the CREB/ATF family, played a pivotal role in the up-regulation of NOX1 in rat VSMCs.

The expression of NOX2 is also known to be modulated by multiple regulatory factors, such as PU.1, E74-like factor 1 (Elf-1), IRF-1, and interferon consensus sequence binding protein in the myelomonocytic cell lineage (312). In addition, a number of DNA-binding proteins also have the capability of interacting with the NOX2 promoter region, including BH3 interacting domain (BID), IRF-1, IRF-2, the CCAAT box binding protein CP1, and the transcriptional repressor CCAAT displacement protein (282, 317-321). It has been shown that IFN-γ and LPS were able to induce the expression of NOX2 in human monocyte derived macrophages and neutrophils (322). TPA, retinoic acid or DMF was also reported to be capable of increasing the NOX2 expression in myeloid cells (317). Moreover, NF-kB-dependent transcription of NOX2 has been reported by Anrather et al (323).

To date there is no report on the mechanism of the inner ear specific expression of NOX3. The expression of NOX3 is almost restricted to the inner ear (225).
NOX4 is constitutively expressed in various cell types. Ubiquitous expression of NOX4 suggests that it acts as a housekeeping gene. Indeed, the promoter region of the NOX4 gene contains many G or C bases, which are characteristics of housekeeping genes (133). On the other hand, the induction of NOX4 has been reported (324). Recently, Diebold et al. (325) have reported that NOX4 mRNA and protein levels were induced in pulmonary artery smooth muscle cells (PASMCs) and pulmonary vessels from mice after exposure to hypoxia. This response was dependent on hypoxia inducible factor-1alpha (HIF-1α), which interacted directly with the NOX4 promoter. Therefore, the induction of NOX4 by HIF-1α may be an important mechanism to maintain ROS levels after hypoxia and the hypoxia induced proliferation of PASMCs. Zhang et al. (326) have demonstrated that growth-promoting transcription factor E2F physically interacts with NOX4 promoter in A7r5 cells and primary mouse aortic smooth muscle cells, thus it provides a new insight into the mechanism of NOX4 gene regulation.

1.3.4.2 Phosphorylation

The phosphorylation of NOX subunits and their assembly into active complexes are mediated by various mechanisms, and many of them are redox-sensitive. It has been demonstrated that a number of stimuli and pathways involved in the activation of NOX by phosphorylation. They comprise phospholipases (PLCβ/γ, PLD), arachidonic acid metabolites, GTP-binding proteins (Ras, Rac1/2), PKC, PI3K, MAPK, and non-receptor protein tyrosine kinases (327, 328). Functional NOX1 has been shown to require Noxo1, Noxa1, and Rac1 (4). Thus, the activation on the subunits and regulatory proteins by phosphorylation can result in an increase in the activity of NOX1. It has been demonstrated that PKA and c-Src mediated phosphorylation of Noxa1 regulates NOX1 activity in human embryonic kidney 293 cells (329, 330). Likewise, NOX2 requires subunits and regulatory proteins for its ROS generation. It has been shown that the phosphorylation regulation of NOX2 is similar to NOX1 (137). NOX4 is constitutively active and does not require phosphorylation of regulatory subunits, whereas NOX5 activity is Ca²⁺-sensitive (137).
Because the phosphorylation status of NOX is tightly controlled by redox sensitive protein kinases and phosphatases, and AP-1, NF-κB, and STAT1/3 are redox sensitive transcription factors, thus, a positive feedback mechanism might exist whereby ROS (possibly generated by NOX) is important for sustained ROS formation in the cardiovascular system. Therefore, it suggests that NOX isoforms themselves are redox regulated at multiple levels.

1.3.5 NOXs in diseases

An accumulating body of evidence shows that enhanced activity and expression of NOX isoforms result in an increase in NOX-derived ROS production, which disturbs the balance between the oxidant and antioxidant system. As a consequence, in vasculature, increased ROS generation causes endothelial dysfunction and initiates a series of events that lead to many cardiovascular pathological states such as atherosclerosis, hypertension, congestive heart failure, ischemia-reperfusion injury, and diabetes associated vascular complications (63, 64). Therefore, the pathophysiological role of NOX isoforms in diseases has been drawn a great deal of attention.

Under physiological condition, NOXs play an important role in regulating embryonic development and wound healing, promoting the transactivation of growth factor receptors, stimulating redox-sensitive signaling cascades, inhibiting phosphatases, activating transcription factors (331-337). In particular, in the vasculature, NOX derived ROS play an role in the maintenance of vascular tone and regulation of cell growth, proliferation, differentiation, apoptosis, cytoskeletal organization, and cell migration (325).

On the other hand, NOX is the primary source of ROS and the main trigger of oxidative stress, it has a pivotal role in the pathology of vasculopathy (338, 339). A significant correlation between the up-regulation of NOX expression and activity and the development of vasculature disease has been reported in patients with cardiovascular and/or metabolic diseases. Table 1.3 summarized the evidence of the participation of NOXs in various diseases.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Isoform</th>
<th>Evidence</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>NOX1, NOX4</td>
<td>+++</td>
<td>NOX1 is a hub in pro-hypertensive signalling</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>NOX1, NOX4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOX2, NOX5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic dissection and aneurysm</td>
<td>NOX1</td>
<td>+++</td>
<td>Oxidative stress in vascular smooth muscle is crucial</td>
</tr>
<tr>
<td>Cardiac hypertrophy, fibrosis, and</td>
<td>NOX2</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>heart failure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>NOX2</td>
<td>+++</td>
<td>Ischemia–reperfusion injury</td>
</tr>
<tr>
<td>Heart transplant</td>
<td>NOX2</td>
<td>+</td>
<td>Ischemia–reperfusion injury</td>
</tr>
<tr>
<td>Doxorubicin cardiotoxicity</td>
<td>NOX2</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>NOX4</td>
<td>+</td>
<td>Both endogenous and pollen oxidase</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary</td>
<td>NOX2</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emphysema</td>
<td>NOX3</td>
<td>+</td>
<td>Upregulation of NOX3 in an emphysema mouse model</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>NOX4</td>
<td>++</td>
<td>NOX4 involved in fibroblast/myofibroblast transition and death of</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>epithelial cells</td>
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<tr>
<td>Pulmonary hypertension</td>
<td>NOX4</td>
<td>++</td>
<td></td>
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<tr>
<td>Pulmonary infections</td>
<td>NOX2</td>
<td>++</td>
<td>NOX2-deficient mice show increased pathogen clearance for influenza</td>
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<td></td>
<td></td>
<td></td>
<td>virus, pneumococci</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>NOX2, NOX1</td>
<td>+++</td>
<td>Binding of mutant SOD1 to Rac1 is involved</td>
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<td>Alzheimer’s disease</td>
<td>NOX2</td>
<td>+++</td>
<td>Microglial NOX2 is important</td>
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<tr>
<td>Parkinson’s disease</td>
<td>NOX2</td>
<td>+++</td>
<td>Microglial NOX2 is important</td>
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<tr>
<td>Schizophrenia</td>
<td>NOX2</td>
<td>+</td>
<td>ROS-dependent loss of phenotype of rapidly spiking interneurons</td>
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<tr>
<td>Ischemic stroke</td>
<td>NOX2</td>
<td>+++</td>
<td>Strong indication</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>NOX4</td>
<td>+++</td>
<td>NOX4 involved in epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>Renal cancer</td>
<td>NOX4</td>
<td>++</td>
<td>NOX4 activates HIF-1α</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>NOX2</td>
<td>+</td>
<td>NOX-containing microparticles might be involved</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>NOX3</td>
<td>++</td>
<td>Cisplatin enhances NOX3-dependent ROS generation</td>
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</table>
### Table 1.3. Continued.

<table>
<thead>
<tr>
<th>Condition</th>
<th>NOX Enzymes</th>
<th>Level of Evidence</th>
<th>Comment</th>
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<tbody>
<tr>
<td>Hepatitis</td>
<td>NOX2</td>
<td>+</td>
<td>Evidence only for alcoholic hepatitis</td>
</tr>
<tr>
<td>Liver ischemia and reperfusion</td>
<td>NOX2</td>
<td>+++</td>
<td>Of interest for liver transplantation?</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>NOX1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Barrett’s esophagus</td>
<td>NOX5</td>
<td>+</td>
<td>Involvement in the development of esophagus cancer?</td>
</tr>
<tr>
<td>Atrophic gastritis</td>
<td>NOX2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>NOX2</td>
<td>+</td>
<td>Due to enhanced or decrease NOX activity?</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>NOX2</td>
<td>+</td>
<td>Due to enhanced or decrease NOX activity?</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>NOX1 NOX2 NOX4</td>
<td>+</td>
<td>NOX enzymes involved in osteoclast differentiation and in bone resorption</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>NOX1 NOX4</td>
<td>+</td>
<td>Overshooting NOX activity in adipocytes</td>
</tr>
<tr>
<td>Diabetes</td>
<td>NOX1 NOX4</td>
<td>+</td>
<td>NOX derived ROS involved in β cell death</td>
</tr>
<tr>
<td>Hyperthyroidism</td>
<td>DUOX2 DUOX1</td>
<td>+++</td>
<td>Severe hypothyroidism in DUOX2-deficient patients</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>NOX4</td>
<td>++</td>
<td>Anti-PDGF receptor antibodies activate NOX in fibroblasts</td>
</tr>
<tr>
<td>UV damage of skin</td>
<td>NOX1</td>
<td>++</td>
<td>UV activates NOX1 in lens keratinocytes</td>
</tr>
</tbody>
</table>

Adapted from Touyz et al (68) and Lambeth et al. (340).

+: preliminary evidence mainly based on indirect data and cellular studies; ++: intermediate level evidence, based at least in part on relevant disease models; +++: strong evidence based on relevant disease models using NOX-deficient mice and/or NOX inhibitors; in some cases also data on human mutations and polymorphisms.

Abbreviation: HIF-1α, hypoxia inducible factor-1alpha; NOX, nicotinamide adenine dinucleotide phosphate oxidase; SOD, superoxide dismutase; UV, ultraviolet.
All this evidence indicates that NOX1, NOX2, NOX4, and NOX5 are important regulators of cellular pathways mediating ROS-dependent pathophysiological processes. Given that the different NOX subtypes are expressed concurrently in the vascular wall cells, and that several isoforms are similarly regulated, their subcellular localization might be an essential factor in determining NOX function (341, 342). In addition to catalytic components, several regulatory subunits, namely p22phox, p40phox, p47phox, p67phox, and Noxa1, have been shown to be up-regulated in vivo and in vitro by vasoactive agents, inflammatory cytokines, growth factors, high glucose, modified lipids and lipoproteins, hyperinsulinemia, homocysteine, and mechanical stress (163, 343-345). Thus, NOXs have a complex role in pathophysiological processes and their activities are subject to regulation in multiple levels by various stimuli.
1.4 eNOS
A family of NOS enzymes catalyzes L-arginine to produce NO and L-citrulline with the requirement of substrate and cofactor. The three mammalian NOS isoforms are coded by different genes but share 50%–60% sequence identity. Although three isoforms share similar chemical and enzymatic properties, different isoforms are featured as distinctive catalytic and regulatory properties. eNOS is one of three distinct isoforms of NOS in mammalian tissues. It is constitutively expressed in ECs and is usually regulated by Ca2+/Calmodulin (CaM) as well as phosphorylation. The other NOS isoforms are neuronal NOS (nNOS), mainly expressed in the brain as well as skeletal muscle, and inducible NOS (iNOS), expression can be induced in macrophages, liver, and vascular smooth muscle (58, 346-348).

1.4.1 Structure and function of eNOS
Human eNOS is encoded by NOS3 and mapped on 7q35–7q36 of chromosome 7 with 1,203 amino acid residues and the molecular weight is 133 kDa. Structurally, all NOS isoforms are homodimers and bidomain enzymes consisting of an N-terminal oxygenase domain (amino acid 1-491) and a C-terminal reductase domain (amino acid 492-1203) (349-351). The N-terminal oxygenase domain includes several binding sites for a prosthetic heme group, BH4, O2, and L-arginine to support the catalytic activity; the C-terminal reductase domain contains several binding sites for FAD, flavin mononucleotide (FMN), and NADPH. These two terminals are linked by CaM recognition site (Figure 1.3) (349, 352, 353).

With regard to the function, all the NOS isoenzymes catalyze the synthesis of NO at the active site requires a stepwise transfer of electrons from C-terminal reductase domain to N-terminal oxygenase domain (Figure 1.3) (354). Within the reductase domain, the electron transfer initiates from NADPH to FAD then to FMN, in which CaM increases the electron transfer and facilitates electron transfer from reductase domain to the heme of the oxygenase domain and convert ferric heme to ferrous. At the heme, the electrons are coupled with L-arginine and reduce O2; NO and L-citrulline are generated in two steps including NOS hydroxylases L-arginine to \(N^G\)-hydroxy-L-arginine and NOS oxidizes \(N^G\)-hydroxy-L-arginine to citrulline and NO (354-356).
Figure 1.3. Functional eNOS.
1.4.2  Pathophysiological role of eNOS
The physiological and pathological role of eNOS has been attracting a great deal of interest from lab and beyond because of the NO generating property. On the other hand, under certain circumstances, eNOS also functions as a ROS generating enzyme causing a series of vicious events. eNOS is constitutively expressed in ECs, and eNOS generated NO is a predominant regulator of endothelial function in endothelium playing a crucial role in regulating vascular tone and homeostasis, in particular, in mediating the expression of pro- and anti-atherosclerotic genes and protecting against thrombosis and atherosclerosis (357).

NO is a free radical gas acting as a second messenger that diffuses rapidly from the endothelium to adjacent cells. It produces vasodilation by the activation of soluble guanylate cyclase, production of cGMP, and ultimately activation of a variety of downstream effectors, including cGMP-dependent protein kinase, cGMP-modulated ion channels, and phosphodiesterases (358). A number of other beneficial actions of NO also have been shown. Endothelium derived NO controls vascular tone and is able to relax blood vessels, prevent platelet aggregation and adhesion, limit oxidation of LDL cholesterol, inhibit proliferation of vascular smooth muscle cells, and decrease the expression of pro-inflammatory genes that advance atherogenesis, inhibits platelet function, prevents adhesion of leukocytes, and reduces proliferation of the intima. (45, 58, 357). An enhanced inactivation and/or reduced synthesis of NO are seen in conjunction with risk factors for cardiovascular disease.

On the other hand, eNOS can become a ROS contributor under certain conditions, which is referred to eNOS uncoupling. It has been implicated in the pathogenesis of many metabolic and cardiovascular diseases, such as diabetes and atherosclerosis. This will be discussed in the eNOS Uncoupling section.
1.4.3 Regulation of eNOS

With an important role in vasculature, the regulation of eNOS is gaining increasing attention. It has been demonstrated that eNOS is subject to modulation at multi-levels including transcription, posttranscription and posttranslation (347, 359).

1.4.3.1 Transcriptional regulation

The transcriptional regulation of eNOS involves multiple *cis* and *trans* regulatory elements which interact with eNOS promoter. Like many other constitutively expressed proteins, eNOS promoter consists of many potential *cis*-regulatory DNA sequences, including a CCAT box, Sp1 sites, GATA motifs, CACCC boxes, AP-1 and AP-2 sites, a p53 binding region, NF-1 elements, acute phase reactant regulatory elements, sterol regulatory elements, and shear stress response elements (360). Moreover, two tightly clustered *cis*-regulatory regions are identified in the human eNOS proximal core promoter and those two regulatory regions are involved in eNOS basal transcription (361). They are positive regulatory domain I and II. In addition to the interaction with *cis*-regulatory elements, it has been shown that basal eNOS promoter activity was also dependent on functional interactions between the *trans*-acting factors that bind to these two regulatory domains (347, 359).

It has been demonstrated that the expression of eNOS mRNA is mainly expressed in the vascular endothelium (362, 363), and the regulation of eNOS mRNA expression has been extensively studied (364). Collectively, it has been demonstrated that the transcriptional regulation of eNOS involves multiple signalling pathways including NF-κB and lung Küppel-like factor pathways (364-367). On the other hand, the repression of eNOS mRNA expression was also reported. Potente et al. (368) revealed that Foxo-1 acted as a transcriptional repressor of eNOS expression. In addition, small RNA regulated eNOS regulation has been described. Zhang et al. (369) reported that a 27-nt derived from 27-nt repeats within intron 4 of the NOS3 gene inhibited eNOS expression.
1.4.3.2 Posttranscriptional regulation

Posttranscriptional regulation is a regulatory process of newly transcribed RNA before it is translated into protein. The process includes modification of primary transcript, nucleocytoplasmic transport, subcellular localization, mRNA stability, and translation efficiency. *Cis*-acting RNA elements is a major regulator of posttranscriptional control in 5'- and 3'-mRNA untranslated regions (5'-UTR and 3'-UTR) (370, 371). A combination of primary and secondary structures of these elements and their recognition by *trans*-acting RNA binding proteins is essential for the posttranscriptional regulation (372).

For eNOS, it has been reported that 3'-UTR is the posttranscriptional regulation site interacting with *cis*-elements (364). Searles et al. (373) reported that a 43-nt sequence located at the origin of the bovine 3'-UTR had a critical role in determining eNOS mRNA stability. The 43-nt region destabilizes eNOS mRNA, however, deletion of this 43-nt region is able to dramatically stabilize a transfected chimeric eNOS mRNA construct (373). In addition, a 25-nt UC-rich sequence, approximately 95 bases distal to the 43-nt sequence, has been reported to be an important modulator for eNOS stability as well (374, 375). For human eNOS mRNA, a CU-rich 158-nucleotide sequence located in the medial portion of the 3'-UTR was identified as being important in regulating mRNA stability (376). The posttranscriptional regulation can also be modulated by growth factor, cytokines and even under pathological conditions. It has been shown that TGF-β1 treatment can increase steady stated eNOS mRNA levels in human ECs (377) and it can augment the expression of eNOS mRNA in a time- and dose-dependent manner in bovine aortic endothelial cells (378). Cyclosporine A treatment has been reported that it can increase the eNOS mRNA expression which may be dependent on the increased H$_2$O$_2$ generation (379). In addition, lysophosphatidylcholine also has been shown to increase ROS production (380) and to increase eNOS transcription (381). Cai et al. (382) also showed that H$_2$O$_2$ up-regulated eNOS expression *via* a calcium/CaM-dependent protein kinase II (CaMKII)-mediated mechanism. On the other hand, it has been described that LPS and TNF-α decreased the steady state levels of eNOS mRNA (359, 373).
1.4.3.3  Posttranslational regulation

The posttranslational regulation of eNOS has been extensively studied, which plays a critical role in controlling the eNOS function and activity (383). Accumulating evidence shows that alteration in eNOS activity and function are conferred by a variety of posttranslational regulatory factors, such as phosphorylation, BH4, heat shock protein (HSP) 90, Ca\(^{2+}\), CaM, and caveolin-1.

1.4.3.3.1  Phosphorylation

There are multiple phosphorylation sites located on eNOS tyrosine (Tyr), serine (Ser), and threonine (Thr) residues, suggesting the potential role of phosphorylation in the regulation of eNOS activity. So far, there are a number of putative phosphorylation sites identified, which include Tyr 81, Tyr567, Ser114, Ser615, Ser633, Ser1177, and Thr495. The most well-known phosphorylation sites with functional consequences are Ser residue (human eNOS sequence: Ser1177: bovine Ser1179) in the reductase domain and a Thr residue (human eNOS sequence Thr495: bovine Thr497) within the CaM-binding domain.

Threonine phosphorylation

Phosphorylation on Thr at the position of 495 is constitutive and it is a negative regulatory site in ECs resulting in a decrease in enzymatic activity (384-386). The negative correlation between Thr495 phosphorylation and NO generation is caused by the interference with the binding of CaM to the CaM-binding domain. It was observed that in ECs stimulated with agonists such as bradykinin, histamine, or Ca\(^{2+}\) ionophore, consequently more CaM binds to eNOS when Thr495 is dephosphorylated (384). The underlying mechanism is a conformational change within eNOS itself, which is induced by the phosphorylation of eNOS at Thr495 resulting in electrostatic repulsion of nearby glutamate residues within CaM and changes in eNOS Glu498 (387). In addition, Lin et al. (388) showed a link between dephosphorylation of Thr495 and eNOS uncoupling.
**Tyrosine phosphorylation**

It has been demonstrated that there are multiple potentially phosphorylatable Tyr residues in eNOS via the observation of attenuation on endothelial NO production and flow induced vasodilatation by Tyr kinase inhibitors (389-391). The tyrosine-phosphorylated residues are Tyr81 and Tyr657 with regulatory effect on NO production (392, 393). Intriguingly, these two residues exert opposite effects on eNOS activity. Tyr81, located in the oxygenase domain, is phosphorylated by v-Src resulting in an increase in the activity of eNOS (392, 393), and it is suggested that the increasing activity of eNOS by Tyr81 phosphorylation is not because of direct modification of eNOS activity but is due to the modulation of the sensitivity of the enzyme to Ca$^{2+}$, alteration of protein-protein interactions or changes in its subcellular localization; Whereas, phosphorylation of Tyr 657 seems to attenuate eNOS activity (394). It is located in the FMN-binding domain and can be stimulated by proline-rich tyrosine kinase 2 (394), fluid shear stress (395) and the activation of Src (396).

**Serine phosphorylation**

Ser phosphorylation is a more considerable regulation of eNOS. There are multiple phosphorylation sites on Ser within eNOS, including Ser1177, Ser633, Ser615 and Ser114. Ser1177 can be rapidly phosphorylated in response to fluid shear stress (397), VEGF (398) and bradykinin (384). In addition, various kinases involved in the Ser1177 phosphorylation includes Akt (399), PKA (400) and AMPK (386, 401). Phosphorylation of Ser1177 plays a major role in the regulation of eNOS activity. It has been shown that hyperglycaemia (402), type 2 diabetes (125) and advanced glycation end products (AGE) (403) resulted in the modification of Ser1177 by O-linked N-acetylglucosylation and thus attenuate eNOS activity and induce an decrease in NO production. Ser633, which is located within one of the auto-inhibitory loops hindering the access of CaM to its binding domain and throttling the activity of eNOS. It is has been reported that the phosphorylation of Ser633 can be regulated by kinases in vitro including PKA and protein kinase G (404, 405), but the functional relevance has not been fully unveiled yet. The phosphorylation of Ser at the position of 615 was identified by phosphopeptide mapping and the phosphorylation of Ser615 has been shown to be stimulated by both PKA and Akt.
Michell et al. (404) observed that mimicking phosphorylation at Ser615 significantly increases the Ca\(^{2+}\)/CaM sensitivity of eNOS, suggesting that it may promote eNOS activity and NO generation. On the other hand, it is suggested that Ser615 may exert an important role in regulating phosphorylation at other sites as well as protein-protein interactions and the assembly of the eNOS signalosome (385, 407). eNOS can also be phosphorylated at Ser114 which is constitutively phosphorylated site and it is proposed to be a negative regulatory site. Indeed, phosphorylation of Ser114 leads to a decrease in eNOS activity (408).

1.4.3.3.2 HSP90

Protein-protein interaction is one of the most important posttranslational regulations of eNOS function and activity. HSP90 is a very important chaperone playing an essential role in modulating the activity and function of NOS. Structurally, it has been reported that HSP90 is required for the folding of NOS enzymes and determines the insertion of heme into the immature protein (409). Functionally, HSP90 participates in numerous signal transduction cascades as a scaffolding molecule. To eNOS, it has been reported that enhanced interaction between HSP90 and eNOS can increase NO yield (410). Presley et al. (411) also showed that activation of HSP90-eNOS interaction induced an increase in NO generation. On the other hand, inhibition of HSP90 is capable of uncoupling eNOS resulting in a decrease in NO production and an increase in ROS generation (412). Disassociation of HSP90 and eNOS impaired the activity of eNOS under hyperglycaemia (413). Similar findings have demonstrated the role of HSP90 in promoting NO generation and inhibiting ROS production as well (414).

There are still various posttranslational regulations of eNOS and some of them have not fully deciphered yet. It has been shown that regulation of catalytic activity of eNOS at the posttranslational level is highly modulated by substrate and cofactor availability, S-glutathionylation, endogenous inhibitors, lipid modification, Ca\(^{2+}\)/CaM, caveolin-1, O-linked glycosylation, and S-nitrosylation (347, 415-417). Disruption of those regulations leads to alterations on eNOS activity and NO output,
which is a drive of numerous pathophysiological disorders including cardiovascular and metabolic diseases.

As described above, eNOS is subject to multiple levels of regulation including transcription, posttranscription and posttranslation. As such, it suggests that eNOS may act as a therapeutic target in preventing or treating vascular diseases. There are a number of clinical trials on the regulation of eNOS via supplementation with eNOS modulators reported (Table 1.4).
<table>
<thead>
<tr>
<th>L-Arginine</th>
<th>Dose</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>With low/high ADMA level</td>
<td>3–8 g·day⁻¹</td>
<td>No acute pharmacological effects. Low ADMA, no effect; high ADMA, normalizes endothelial function</td>
<td>(418)</td>
</tr>
<tr>
<td>Post-MI</td>
<td>3.0 g po, 3 times daily, for 6 mon</td>
<td>No serious adverse effects, possibly associated with higher mortality, no improvement of vascular stiffness measurements, left ventricular ejection fraction</td>
<td>(419)</td>
</tr>
<tr>
<td>Peripheral arterial disease</td>
<td>3 g·day⁻¹ po for 6 mon</td>
<td>No improvement of vascular reactivity, no benefit, possible harm</td>
<td>(420)</td>
</tr>
<tr>
<td>FA/5-MTHF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5-MTHF, 1 µg·100 ml FAV⁻¹·min⁻¹, local forearm intra-arterial</td>
<td>Improvement of endothelial dysfunction</td>
<td>(421)</td>
</tr>
<tr>
<td>Hyperhomocysteinemia</td>
<td>FA, 10 mg·day⁻¹ po for 8 wk</td>
<td>Improvement of endothelium-dependent dilation, higher serum folate levels, lower total plasma homocystine levels</td>
<td>(422)</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>5-MTHF 0.13 mg·kg⁻¹ body wt</td>
<td>Improved NO-mediated endothelium-dependent vasomotor responses, reduced vascular superoxide</td>
<td>(423)</td>
</tr>
<tr>
<td>6R-BH4 (analogue of BH4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>5 mg·kg⁻¹ po, twice daily for 8 wk</td>
<td>Not statistically significant. Drop of 6.4 mmHg in patients’ SBP</td>
<td>2008 BioMarin Pharmaceutical, NCT00325962 (unpublished)</td>
</tr>
<tr>
<td>Condition</td>
<td>Description</td>
<td>Outcome</td>
<td>Source</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension</td>
<td>2.5 mg·kg(^{-1})·day(^{-1}) for 2 wk, 5 mg·kg(^{-1})·day(^{-1}) for 2 wk, 10 mg·kg(^{-1})·day(^{-1}) for 4 wk, and then 20 mg·kg(^{-1})·day(^{-1}) for 2 days</td>
<td>Unpublished, ongoing Vanderbilt University, The National Institutes of Health, GCRC&amp; BioMarin Pharmaceutical, NCT00435331 (unpublished)</td>
<td></td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<td>5 mg·kg(^{-1}) po, twice daily for 13.5 days</td>
<td>Not significant.</td>
<td>2009 BioMarin Pharmaceutical, NCT00532844 (unpublished)</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>16-wk dose escalation phase po, every 4 wk as follows: 2.5, 5, 10 (once daily), and 20 mg·kg(^{-1})·day(^{-1}) (twice daily), and continued in an optional extension phase at the highest tolerated dose for up to a total of 2 yr</td>
<td>Improvement of endothelial function</td>
<td>2009 BioMarin Pharmaceutical, NCT00445978 (unpublished)</td>
</tr>
<tr>
<td>Peripheral arterial disease</td>
<td>400 mg po, twice daily for 24 wk</td>
<td>Not significant.</td>
<td>2009 BioMarin Pharmaceutical, NCT00403494 (unpublished)</td>
</tr>
</tbody>
</table>

Adapted from Zhang et al. (424).

Abbreviation: 5-MTHF, 5-methyltetrahydrofolate; ADMA, asymmetric dimethyl-L-arginine; BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; FA, folic acid; FAV, forearm volume; MI, myocardial infarction; SBP, systolic blood pressure.
1.4.4 eNOS Uncoupling

As we have described above, eNOS is a critical enzyme for the maintenance of vascular homeostasis. It catalyzes the formation of NO from L-arginine and O_2 in a reaction requiring adequate substrates and cofactors including Ca^{2+}/CaM, FAD, FMN, NADPH, and BH4. Electrons are transported through the reductase domain via NADPH, FAD and FMN in functional homodimerized form (58, 425, 426). However, under certain circumstances, eNOS can be uncoupled. The electron flown from the FMN group to the ferrous-dioxygen heme complex on the adjacent oxygenase domain is disturbed so that electrons are diverted to O_2 leading to O_2^- production, and thus eNOS turns out to be O_2^- generating enzyme (Figure 1.4) (427, 428). In uncoupled state, NADPH consumption and O_2 reduction are uncoupled from L-arginine oxidation and NO formation with subsequently decreased NO production and increased O_2^- generation (429-431). A further implication of this progressive eNOS uncoupling is that eNOS produces both NO and O_2^-, which form ONOO^-.

Thereby eNOS effectively becomes an ONOO^- generating enzyme (432). ONOO^- has significant implications both as a contributor to vascular oxidative stress per se, and also as a damaging factor of further BH4 depletion and eNOS uncoupling.
Figure 1.4. eNOS uncoupling.
eNOS uncoupling is a mechanism resulting in endothelial dysfunction. Accumulating evidence shows the association between eNOS uncoupling and endothelial dysfunction in patients with hypercholesterolemia (433), diabetes (434), essential hypertension (435); chronic smokers (436); and in nitroglycerin treated patients (437). As such, the pathological risk factors which will lead to eNOS uncoupling is proposed to be responsible for the development of endothelial dysfunction in vascular disease. Therefore, it suggests that the approaches which enable the restoration of eNOS uncoupling is capable of improving endothelial dysfunction.

1.4.4.1 Regulation of eNOS uncoupling

Accumulating evidence suggests that eNOS uncoupling has been implicated in many cardiovascular and metabolic diseases with shift of nitroso-redox balance. Under physiological conditions, eNOS requires BH4 and L-arginine to generate NO. Under inadequate cofactors conditions, eNOS mediated O2 reduction no longer couples to L-arginine, resulting in the generation of O2− rather than NO. This eNOS uncoupling contributes to increased ROS production and decreased NO formation, consequently endothelial dysfunction (438). An increasing body of evidence shows that eNOS uncoupling can be modulated by directly or indirectly increasing the bioavailability of its cofactors and substrate, increasing its transcription and interfering with eNOS modulators, such as netrin-1, resulting in increased NO production and/or less eNOS-dependent generation of free radicals.

1.4.4.1.1 BH4

BH4 (5,6,7,8-tetrahydrobiopterin) is an essential cofactor of enzymes that are of physiological importance, including three NOS isoenzymes (416). It is synthesized via de novo synthesis and salvage pathway (Figure 1.5). BH4 is widely present in cells or tissues of higher organisms and plays a key role in a number of biological processes and pathological states, such as monoamine neurotransmitter formation, endothelial dysfunction, the immune response and pain sensitivity (439).

With regards to eNOS, the NO generating function is highly correlated with the intracellular concentration of BH4 (439-441). In coupled state, BH4 is tightly bound
to eNOS and facilitates eNOS to catalyze the formation of L-citrulline and NO in the presence of its adequate substrates (L-arginine and oxygen) through easing the binding of L-arginine substrate to eNOS, shifting the heme iron into a high spin state to increase enzyme activity, involving in electron transfer, and stabilizing the NOS dimer (442-445). Whereas, in the presence of inadequate levels of BH4, the pleiotropic role of BH4 in NO generation is impaired and the electron transfer in eNOS becomes uncoupled from L-arginine oxidation and NO formation to form $O_2^-$. Moreover, inadequate bioavailability of BH4 causes a less tightly packed oxidase domain of eNOS dimer and a higher sensitivity to proteolysis (446).
BH4 biosynthesis initiates from GTP via 7,8-dihydroneopterin (BH2) and 6-pyruvoyl-tetrahydropterin. The first and rate-limiting step in de novo pathway is catalysation by guanosine triphosphate cyclohydrolase I (GTPCH1). Subsequent steps are catalyzed by the enzymes 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase. In salvage pathway, whereby 6-pyruvoyl-5,6,7,8-tetrahydropterin is converted to sepiapterin by an enzyme termed “sepiapterin synthase.” Sepiapterin is reduced by sepiapterin reductase to BH2, and further by dihydrofolate reductase (DHFR) to form BH4.
Accumulating evidence shows that reduced BH4 bioavailability lead to eNOS uncoupling and endothelial dysfunction (439). Conversely, in many of these conditions, supplementation with exogenous BH4 has been shown to improve endothelial function and lower cardiovascular risks. It has been shown that BH4 supplementation diminished the NOS-dependent generation of $\text{O}_2^-$ (447), and restored endothelial function in diabetic animal models of diabetes (448), insulin resistance (449), as well as in patients with hypercholesterolemia (433), diabetes (434), hypertension (435), and in chronic smokers (436). Moreover, it has been demonstrated that BH4 improved ischemia-reperfusion injury in isolated perfused rat hearts (430, 450). In ApoE−/− mice, BH4 supplementation reduced vascular immune cell infiltration and prevented the progression of atherosclerosis through maintaining eNOS coupling and NO bioavailability (451). In addition, exogenous BH4 given to mice can recouple an already uncoupled eNOS with a subsequent reversing of pressure overload induced cardiac hypertrophy, fibrosis, and myocardial dysfunction (452).

On the other hand, it has been shown that up-regulation of BH4 synthesis produced beneficial effect on endothelial function. Ozaki et al. (453) showed that upregulation of the rate-limiting enzyme of BH4 synthesis, GTPCH1, led to a recovery of eNOS bioactivity, thereby improving endothelial function and reducing disease progression. Zhang et al. (454) reported that the increased expression of GTPCH1 prevented the decrease in BH4 levels in isolated cells and in atherosclerotic animal models. In addition, Crabtree et al. (455) showed that increased expression and activity of DHFR prevented eNOS from uncoupling. Conversely, inhibition of GTPCH1 resulted in eNOS uncoupling (456).

1.4.4.1.2 L-Arginine

Reduced availability of L-arginine has been suggested as a contributor of NOS uncoupling (457, 458). The beneficial effects of L-arginine supplement have been reported in both animal and human studies under a variety of pathophysiological conditions such as in patient with hypercholesterolemia (459), patients with essential and secondary hypertension (460), in vessels (461, 462). It has been shown that
short- to medium-term administration of arginine improves NO mediated vascular function in elderly humans as well as a variety of clinical conditions, including hypercholesterolemia, coronary artery disease, congestive heart failure, and peripheral artery disease (418). Furthermore, it has been reported that L-Arginine is capable of stimulating $O_2$ uptake by coupled eNOS (463), inhibiting $O_2^-$ formation within uncoupled eNOS by electronic interaction with the heme-bound $O_2$ (464). However, Non-beneficial effect of L-arginine was also observed in other trials (418) or the benefits did not continue with chronic therapy. This raises the question whether L-arginine concentrations can become critical as a substrate \textit{in vivo}.

Excessive arginase activity induced low L-arginine bioavailability in the vicinity of eNOS could partially explain the outcome of L-arginine supplementation (58). Moreover, competition of ADMA might be another explanation (465).

1.4.4.1.3 Dimerization

The dimeric form of eNOS is critical for eNOS activity and the ratio of eNOS dimer to monomer has been proposed to be a clue to eNOS uncoupling (Figure 1. 4B). The active form of eNOS enzyme comprises of two identical subunits that form a head to tail homodimer. Cys94 and Cys99 of eNOS form a zinc tetra-coordinated cluster ($\text{ZnS}_4$) between each subunit. It has been shown that zinc bound to the tetrathiolate cluster stabilizes the dimer interface on the N-terminal region of eNOS (466, 467). $\text{ZnS}_4$ is highly sensitive to oxidants such as ONOO$^-$ (53, 468, 469), and oxidation of the $\text{ZnS}_4$ cluster results in monomerization of eNOS and inhibition of catalytic activity. Indeed, the uncoupling of eNOS has been associated to its monomerization. It has been shown that ONOO$^-$ treatment induces the monomerization of eNOS and thus reduces eNOS activity in ECs (53). Cai et al. (470) showed an enhance in eNOS homodimerization by increasing BH4 bioavailability. Whereas, it has been demonstrated that monomerized eNOS continued to produce $O_2^-$ (471, 472). This would lead to the formation of reactive iron-oxygen complex within the heme and highly oxidative heme states, which in turn could result in oxidation of surrounding proteins with further self-amplification of oxidative damage. Thus, dimerization can play a important role in NO production and endothelial function.
1.5 Approaches to improve endothelial function

Endothelial function is critical to maintain the vascular homeostasis. It is well known that endothelial function correlates with cardiovascular outcomes which can be predicted by the grade of endothelial function. On the other hand, endothelial dysfunction initiates the pathogenesis of vascular diseases including atherosclerosis with characterization of reduced NO production and increased ROS generation. Thus, in order to improve endothelial function and prevent or treat vascular diseases, approaches to increase NO availability and reduce ROS level have emerged. There are two ways to eliminate the excessive ROS, one is by scavenging either through the supplement of antioxidants or stimulation of endogenous antioxidant strategy; the other one is targeting on the enzymes involved in the process of ROS production to block the ROS generation. To increase NO availability can be achieved by enhancing the expression of NOS, increasing the activity of NOS and reducing NO degradation.

1.5.1 Vitamins

Vitamins, including vitamin A, C, and E, are the most commonly used antioxidant to remove excessive ROS (473). Their antioxidant activity has been demonstrated both in vitro and in vivo studies (474, 475). The large clinical trials of antioxidants have also been conducted (Table 1.5). The beneficial effect of vitamin A has been demonstrated. Singh et al. (476) reported that vitamin A exhibited protective effect against cardiac necrosis and oxidative stress. It also has been demonstrated that vitamin C provided protection against oxidative stress induced cellular damage by scavenging ROS, protecting proteins from alkylation by electrophilic lipid peroxidation products (477). Vitamin C also played a role in regulating eNOS function by recycling the eNOS cofactor, BH4, which was relevant to arterial elasticity and blood pressure regulation (478, 479). Furthermore, vitamin E (α-tocopherol) functioned as an essential lipophilic antioxidant scavenging hydroperoxyl radicals in a lipid milieu (475). It is suggested that vitamin E plays an important role in protecting erythrocyte membranes and nervous tissues by its antioxidant properties (475). Supplementation of antioxidant seems to be effective on scavenging excessive ROS and relieve oxidative stress in some cases, however, under certain circumstances, administration of antioxidant does not produce beneficial
effects (480). This may be due to the different stages of diseases and the size of the trial.
<table>
<thead>
<tr>
<th>Clinical trials</th>
<th>Dosage</th>
<th>Effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATBC</td>
<td>(\alpha)-tocopherol (50 mg). Daily.</td>
<td>Marginal effect on the incidence of fatal coronary heart disease in male smokers with no history of myocardial infarction for median follow-up of 6.1 years.</td>
<td>(481)</td>
</tr>
<tr>
<td>ASAP</td>
<td>91 mg (136 IU) of D-(\alpha)-tocopherol and 250 mg of slow-release vitamin C. Twice daily.</td>
<td>Significant retard of progression of common carotid atherosclerosis in men. No effect in women and lower atherosclerosis inhibition by individual vitamins use for three years.</td>
<td>(482)</td>
</tr>
<tr>
<td>CLAS</td>
<td>Vitamin E ((\geq) 100 IU), vitamin C ((\geq) 250 mg). Daily.</td>
<td>Less carotid atherosclerosis progression among nonsmoking men after coronary artery bypass graft surgery was found for high supplementary vitamin E use when compared with low vitamin E use. No effect of vitamin E with colestipol/niacin, as well as no effect of vitamin C with colestipol/niacin or placebo was found.</td>
<td>(483)</td>
</tr>
<tr>
<td>CHAOS</td>
<td>(\alpha)-tocopherol in the form of capsule containing 800 IU daily for first 546 patients; 400 IU daily for remainder.</td>
<td>Reduction of non-fatal myocardial infarction in patients with angiographically proven coronary atherosclerosis. Non-significant excess of cardiovascular deaths.</td>
<td>(484)</td>
</tr>
<tr>
<td>Fang et al.</td>
<td>Vitamin C (500 mg) plus vitamin E (400 IU), each twice daily.</td>
<td>Retardation of the early progression of transplant-associated coronary arteriosclerosis after cardiac transplantation.</td>
<td>(485)</td>
</tr>
<tr>
<td>IEISS</td>
<td>Vitamins A (50,000 IU), vitamin C (1000 mg), vitamin E (400 mg), (\beta)-carotene (25 mg). Daily.</td>
<td>Lower infarct size and oxidative stress in patients with suspected acute myocardial infarction on combined treatment.</td>
<td>(476)</td>
</tr>
<tr>
<td>SPACE</td>
<td>Vitamin E (800 IU). Daily.</td>
<td>Reduction of composite cardiovascular disease endpoints and myocardial infarction in haemodialysis patients with prevalent cardiovascular disease.</td>
<td>(486)</td>
</tr>
</tbody>
</table>
Table 1. Continued.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Intervention</th>
<th>Outcome</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WACS</td>
<td>Vitamin E (600 IU every other day); ascorbic acid (500 mg/day) and vitamin E (600 IU every other day).</td>
<td>Marginally significant reduction in the combined outcome of myocardial infarction, stroke, coronary revascularization, or CVD death among women with prior CVD with active vitamin E treatment was observed. Fewer strokes were experienced in female with a history of CVD or 3 or more CVD risk factors with supplementation of ascorbic acid and vitamin E for ~9.4 years.</td>
<td>(487)</td>
</tr>
<tr>
<td>No effect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATBC</td>
<td>β-carotene (20 mg), α-tocopherol (50 mg). Daily.</td>
<td>No primary preventive effect in male smokers with no history of myocardial infarction of 20 mg/day of β-carotene on major coronary events and no influence of α-tocopherol on nonfatal myocardial infarction for median follow-up of 6.1 years. No significant differences in the number of major coronary events in male smokers with previous myocardial infarction receiving α-tocopherol and/or β-carotene for median follow-up of 5.3 years.</td>
<td>(481, 488)</td>
</tr>
<tr>
<td>CARET</td>
<td>β-carotene (30 mg) and vitamin A (25,000 IU). Daily.</td>
<td>No benefits in smokers, former smokers and workers exposed to asbestos from combination of β-carotene and vitamin A in the form of retinyl palmitate for ~4.0 years.</td>
<td>(480)</td>
</tr>
<tr>
<td>GISSI</td>
<td>α-tocopherol (300 mg). Daily.</td>
<td>No benefit in patients surviving recent (≤3 months) myocardial infarction.</td>
<td>(489)</td>
</tr>
<tr>
<td>HATS</td>
<td>D-α-tocopherol, (400 IU), vitamin C (500 mg) and β-carotene (12.5 mg of natural β-carotene) and selenium (50 µg). Twice daily.</td>
<td>No significantly lower atherosclerosis progression in patients with coronary disease and low plasma levels of HDL. Attenuation of benefits from simvastatin-niacin treatment.</td>
<td>(490)</td>
</tr>
<tr>
<td>HOPE</td>
<td>Vitamin E (400 IU).</td>
<td>No apparent effects in patients at high risk for cardiovascular events vitamin E for ~4.5 years treatment.</td>
<td>(491)</td>
</tr>
<tr>
<td>HPS</td>
<td>Vitamin E (600 mg) vitamin C (250 mg) and β-carotene (20 mg). Daily.</td>
<td>No significant reductions in mortality from or incidence of any type of vascular disease, cancer or other major outcome among adults with coronary disease, other occlusive arterial disease or diabetes for 5 years.</td>
<td>(492)</td>
</tr>
<tr>
<td>MICROHOP</td>
<td>Vitamin E (400 IU). Daily.</td>
<td>No effect on cardiovascular outcomes or nephropathy in middle-aged and elderly people with diabetes and CV disease and/or additional coronary risk factor(s) for ~4.5 years.</td>
<td>(493)</td>
</tr>
<tr>
<td>Trial</td>
<td>Vitamin supplementation</td>
<td>Summary</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MVP</td>
<td>β-carotene (30,000 IU), vitamin C (500 mg), vitamin E (700 IU), all given twice daily for four weeks before, and six months after angioplasty with extra vitamin E (2000 IU) 12 h before angioplasty.</td>
<td>No significant reduction in luminal diameter reduction and restenosis rates per segment in patients after angioplasty. Attenuation of beneficial probucol effects.</td>
<td>(494)</td>
</tr>
<tr>
<td>PHS</td>
<td>β-carotene (50 mg). Every other day.</td>
<td>No early or late differences in the overall incidence of cardiovascular disease, malignant neoplasms or death from all causes among healthy men for 12 years.</td>
<td>(495)</td>
</tr>
<tr>
<td>PPP</td>
<td>Vitamin E (300 mg). Daily.</td>
<td>No beneficial effects in primary prevention of cardiovascular events in people.</td>
<td>(496)</td>
</tr>
<tr>
<td>SCPS</td>
<td>β-carotene (50 mg). Daily.</td>
<td>No support for a strong effect in reducing mortality from cardiovascular disease or other causes during median follow-up of 8.2 years.</td>
<td>(497)</td>
</tr>
<tr>
<td>SU.VI.MAX</td>
<td>Vitamin C (120 mg), vitamin E (30 mg), β-carotene (6 mg), selenium (100 µg), and zinc (20 mg). Daily.</td>
<td>No beneficial effects on carotid atherosclerosis and arterial stiffness in free of symptomatic chronic diseases and apparently healthy people for over ~7.2 years.</td>
<td>(498)</td>
</tr>
<tr>
<td>VEAPS</td>
<td>D/L-α-tocopherol (400 IU). Daily.</td>
<td>Null results to the IMT progression in people at low risk for CVD.</td>
<td>(499)</td>
</tr>
<tr>
<td>WACS</td>
<td>Ascorbic acid (500 mg/d), vitamin E (600 IU every other day) or β−carotene (50 mg every other day)</td>
<td>No overall effect on the myocardial infarction, stroke, coronary revascularization, or CVD death considered single or combined in female with a history of CVD or 3 or more CVD risk factors for ~9.4 years.</td>
<td>(487)</td>
</tr>
<tr>
<td>WAVE</td>
<td>Vitamin E (400 IU) plus vitamin C (500 mg). Twice daily.</td>
<td>No cardiovascular benefit in postmenopausal women with coronary stenosis. Potential for harm.</td>
<td>(500)</td>
</tr>
<tr>
<td>Negative results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATBC</td>
<td>β-carotene (20 mg), or combination of α-tocopherol (50mg) and β-carotene (20 mg). Daily.</td>
<td>Increased risk of fatal coronary heart disease in male smokers with previous myocardial infarction for median follow-up of 5.3 years.</td>
<td>(488)</td>
</tr>
</tbody>
</table>
**Table 1. 5. Continued.**

<table>
<thead>
<tr>
<th>CARET</th>
<th>β-carotene (30 mg) and vitamin A (25,000 IU). Daily.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>May have had an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer, cardiovascular disease and any cause in smokers, former smokers and workers exposed to asbestos from combination for ~4.0 years. (480)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WAVE</th>
<th>Vitamin E (400 IU) and vitamin C (500 mg). Twice daily.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increased risk of death in postmenopausal women with coronary stenosis. (500)</td>
</tr>
</tbody>
</table>

Adapted from Badimon et al. (501).

Abbreviation: ATBC, Alpha-Tocopherol & Beta-Carotene Trial; ASAP, Antioxidant Supplementation in Atherosclerosis Prevention Study; CLAS, Cholesterol Lowering Atherosclerosis Study; CHAOS, Cambridge Heart Antioxidant Study; IEISS, Indian Experiment of Infarct Survival Study; SPACE, Secondary Prevention with Antioxidants of Cardiovascular disease in Endstage renal disease; WACS, Women's Antioxidant and Cardiovascular Study; CARET, β-Carotene and Retinol Efficacy Trial; GISSI, Gruppo Italiano per lo Studio della Sopravvivenza nell' Infarto miocardico; CVD, cardiovascular disease; HATS, HDL-Atherosclerosis Treatment Study; HDL, high density lipoprotein; HOPE, Heart Outcomes Prevention Evaluation Study; HPS, Heart Protection Study; MICRO-HOPE, Microalbuminuria, Cardiovascular, and Renal Outcomes in the HOPE study; MVP, MultiVitamins & Probucol Trial; PHS, Physicians' Health Study; PPP, Primary Prevention Project; SCPS, Skin Cancer Prevention Study; SU.VI.MAX, Supplementation in Vitamins and Mineral Antioxidants trial; VEAPS, Vitamin E Atherosclerosis Prevention Study; WAVE, Women's Angiographic Vitamin and Estrogen Trial.
1.5.2 NADPH inhibitor

As described before, NOXs are the primary source of ROS in vasculature contributing to oxidative stress and consequently resulting in eNOS uncoupling, which renders NOXs to be a putatively potential therapeutic target to alleviate oxidative stress and restore eNOS uncoupling. It has been described that combating oxidative stress in vascular diseases by targeting NOX families enables the development of novel drugs to prevent and/or treat vascular diseases. A number of studies have been shown that inhibition of NOXs can improve endothelial function (213, 308, 340, 502-506). In addition, there are various NOXs gene depletion model showing the effect of regulation of NOXs. And there are a number of established and novel NOXs inhibitors identified (Table 1.6), which suggests that regulation on the activity and/or expression of NOXs is a practical approach to tackle excessive ROS and improve endothelial function.
**Table 1.6. Mechanism of action and effects of NOXs inhibitors.**

<table>
<thead>
<tr>
<th>Class of inhibitor</th>
<th>Name</th>
<th>Chemical structure</th>
<th>Mechanism of action</th>
<th>Pharmacological effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-peptide</td>
<td>DPI</td>
<td><img src="image" alt="DPI molecule" /></td>
<td>Flavin containing protein inhibitor - abstracts electrons from FAD and prevents electron flow through the flavocytochrome conduit.</td>
<td>Inhibitor of NADH: ubiquinone oxidoreductase, NADH dehydrogenase, XO, CYP450, NOS, bacterial nicotine oxidase.</td>
<td>(507-512)</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>GK-136907</td>
<td><img src="image" alt="GK-136907 molecule" /></td>
<td>Purported NOX1 and NOX4 oxidase inhibitor. Mechanism of action not defined. But structural similarity with NADPH suggests that it may act as a competitive substrate inhibitor of this enzyme.</td>
<td>None reported.</td>
<td>(513, 514)</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>ML171</td>
<td><img src="image" alt="ML171 molecule" /></td>
<td>A phenothiazine compound with selectivity for NOX1 oxidase over other NOXs. Does not scavenge ROS generated by xanthine oxidase activity.</td>
<td>None reported.</td>
<td>(515)</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>VAS3947</td>
<td><img src="image" alt="VAS3947 molecule" /></td>
<td>A triazolopyrimidine that reduced NOXs derived ROS generation in several cell lines with low micromolar potency, irrespective of the specific isoforms expressed; showed no inhibitory effects against xanthine oxidase derived ROS and eNOS activity.</td>
<td>None reported.</td>
<td>(516)</td>
</tr>
<tr>
<td>Table 1. 6. Continued.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non-peptide</strong></td>
<td>Apocynin</td>
<td><img src="image" alt="Apocynin" /></td>
<td>Oxidase assembly inhibitor-inhibits association of p47phox with membrane heterodimer.</td>
<td>Scavenger of H$_2$O$_2$.</td>
<td>(517-519)</td>
</tr>
<tr>
<td><strong>Peptide</strong></td>
<td>Gp91dstat</td>
<td>[H]-RKKRRQRRRCSTRIRQL-NH$_2$</td>
<td>Oxidase assembly inhibitor-inhibits association of NOX2 with p47phox. Does not scavenge superoxide generated by cell free systems.</td>
<td>No other reported effects.</td>
<td>(260, 520)</td>
</tr>
<tr>
<td><strong>Non-peptide</strong></td>
<td>AEBSF</td>
<td><img src="image" alt="AEBSF" /></td>
<td>Oxidase assembly inhibitor-inhibits association of NOX2 with p47phox. Does not scavenge superoxide generated in cell free systems.</td>
<td>Non-selective serine protease inhibitor.</td>
<td>(521)</td>
</tr>
<tr>
<td><strong>Peptide</strong></td>
<td>PR-39</td>
<td>RRRPRPPYLPFRPFPFPRRL PPFPGRPFPFPPFFP</td>
<td>Binds to SH3 domains of the p47phox subunit and prevents binding to p22phox.</td>
<td>Not selective for NOX as it is likely to inhibit other proteins containing SH3 domains.</td>
<td>(522)</td>
</tr>
<tr>
<td><strong>Non-peptide</strong></td>
<td>Statins (e.g. mevastatin)</td>
<td><img src="image" alt="Statins" /></td>
<td>Decrease superoxide production by inhibiting synthesis of farnesylpyrophosphate and geranylgeranylpyrophosphate which are crucial for membrane attachment of Rac and NOX assembly. May also decrease p22phox and NOX1 expression. Likely to influence NOX1 and NOX 2 activities.</td>
<td>HMG-CoA reductase inhibitor. Decreases AT1 receptor expression; increases eNOS expression.</td>
<td>(523, 524)</td>
</tr>
</tbody>
</table>
**Table 1. 6. Continued.**

<table>
<thead>
<tr>
<th>Non-peptide</th>
<th>AT1 receptor antagonists (e.g. losartan)</th>
<th>Decrease Ang II-dependent activation of NOXs via AT1 receptors. Unlikely to display NOX selectivity as Ang II stimulates NOX1 and NOX4 oxidases.</th>
<th>(107)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-peptide</td>
<td>Nebivolol</td>
<td>Inhibits membrane association and also interaction of p67phox and Rac and decreases oxidase expression. Inhibits NOX1-dependent superoxide production.</td>
<td>β-adrenoceptor blocker.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Resveratrol</td>
<td>Decreases superoxide production in intact macrophages and homogenates. Does not scavenge superoxide in cell free systems.</td>
<td>Inhibitor of PKC.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Gliotoxin</td>
<td>A fungal metabolite, thiol-modifying toxin thought to inhibit phosphorylation of p47phox by preventing PKC co-localization with p47phox. Also, inhibits electron transport through the flavocytochrome before oxidase activation. Low potency for blocking NOX4.</td>
<td>Stimulation of cGMP release. Cytoskeletal re-organisation. Disrupts the mitochondrial membrane potential.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>S17834</td>
<td>Flavonoid derivative proposed to directly inhibit NOXs activity; although the mechanism is still undefined.</td>
<td>No other reported effects.</td>
</tr>
<tr>
<td>Peptide</td>
<td>Clostridium difficile toxin B</td>
<td>For structure see Jank et al. (537).</td>
<td>Glucosylation of threonine-35 on Rac, which modifies GTPase activity.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Nordihydroguaiaretic acid</td>
<td>Blocks H₂O₂ production in macrophages in response to phorbol esters and in ECs in response to thrombin.</td>
<td>Lipoxygenase inhibitor. Blocks arachidonic acid metabolism.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>SKF525A</td>
<td>Decreases superoxide and H₂O₂ production in ECs.</td>
<td>CYP inhibitor.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Metformin</td>
<td>Scavenges hydroxyl radicals but not superoxide. Could also inhibit PMA and Ang II-dependent ROS production from NOXs. However, this is likely to be due to inhibition of PKC activity.</td>
<td>Antihyperglycemic agent. PKC inhibitor.</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Sildenafil-citrate</td>
<td>Inhibits endothelial superoxide production and gp91phox expression in response to the thromboxane mimetic U46619 via increases in cGMP.</td>
<td>An inhibitor of phosphodiesterase type 5. Unlikely to be selective for any particular isoform of NOX or a direct inhibitor of NOX enzyme. Its PDE inhibitory actions are likely to influence many cellular systems. Changes in gp91phox expression could influence immune system.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Bilirubin</td>
<td>Can inhibit superoxide production in neutrophil lysates when applied to cytosolic fractions prior to reconstitution with membrane fractions and SDS stimulation. Unlikely to influence expression of NOX2, p22phox and p47phox but may reduce p47phox phosphorylation.</td>
<td>May also scavenge ROS.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Minocycline</td>
<td>A tetracycline derivative that inhibits NOX derived superoxide production in microglia and dopaminergic neurons in response to stimuli such as thrombin by down-regulating p67phox expression.</td>
<td>Antibiotic.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Perhexiline</td>
<td>Inhibits superoxide production in intact neutrophils stimulated by FMLP or PMA, by an undefined mechanism. Does not inhibit enzyme assembly.</td>
<td>Efficacious anti-anginal agent that inhibits carnitine-palmitoyl-transferase.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Roxithromycin</td>
<td>Blocks superoxide generated by intact neutrophils activated by FMLP or PMA but not by cell lysates. Does not inhibit PKC-dependent phosphorylation. May inhibit translocation of p47phox and/or p67phox to the plasma membrane.</td>
<td>Antibiotic belonging to the macrolide class. Inhibit RNA-dependent protein synthesis. Capable of inhibiting CYP450.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Taurine chloramine</td>
<td>Reversible inhibition of PMA-dependent superoxide anion production in human neutrophils by interfering with the translocation of p47phox and p67phox to the membrane. Also inhibits phosphorylation of p47phox.</td>
<td>Inhibits inducible NOS in alveolar macrophages.</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Peptide</td>
<td>Ghrelin</td>
<td>GSSFLSEHQRVQRLESLLPPAKLQPR</td>
<td>Inhibits superoxide production by thoracic aorta most probably via release of NO. Does not scavenge superoxide.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>α-tocopherol</td>
<td></td>
<td>Decreases stimulated superoxide production, inhibits p67phox-p47phox translocation and p47phox phosphorylation in monocytes, neutrophils and microglial cells. This effect is likely to be due to PKC inhibition.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Benzylisothiocyanate (BITC)</td>
<td>Concentration-dependently blocks TPA induced superoxide production in a human leukemia cell line without influencing PKC activity and p47phox translocation. Mechanism may involve covalent cysteine modification of the NOX.</td>
<td>May inhibit NO, PGE2 and TNF-α production. Can cause apoptosis via induction of Bak and Bax proteins.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Probucol</td>
<td>Decreases superoxide production in rabbit aorta, which may be attributed to down-regulation of p22phox.</td>
<td>Free radical scavenger.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Plumbagin</td>
<td>Inhibits NAD(P)H-dependent superoxide production in various cell lines that express NOX4. Mechanism of action is unknown.</td>
<td>Napthoquinone structure may impart ROS-scavenging effects.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>VAS2870</td>
<td>Undefined mechanism of action. Inhibits NOX activity in NOX2-containing HL-60 cell line and in vascular ECs containing NOX2 and NOX4. Does not scavenge superoxide.</td>
<td>No other reported effects.</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Nitrolinoleate</td>
<td>A nitrated lipid which inhibits PMA- and FMLP-dependent superoxide production and degranulation in human neutrophils by increasing cAMP but not cGMP levels.</td>
<td>Other effects associated with raising cAMP. Vasorelaxation.</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Non-peptide</td>
<td>Mycophenolate acid</td>
<td>A fungal derivative that inhibits endothelial and neutrophil derived superoxide by reducing Rac levels and activation in the membrane. Does not alter mRNA levels of NOX2, NOX4 and p47phox.</td>
<td>A potent inhibitor of inosine monophosphate dehydrogenase involved in purine synthesis in B and T-lymphocytes.</td>
</tr>
</tbody>
</table>

(585, 586) (587, 588)
Table 1. 6. Continued.

<table>
<thead>
<tr>
<th>Non-peptide</th>
<th>Curcurmin</th>
<th>Decreases superoxide production in intact macrophages and homogenates. Does not scavenge superoxide in cell free system.</th>
<th>Is a potent irreversible inhibitor of thioredoxin reductase via alkylation of cysteine residues?</th>
</tr>
</thead>
</table>

Abbreviation: AEBSF, 4-(2-aminoethyl)-benzenesulphonyl fluoride; Ang II, angiotensin II; AT1 receptor, angiotensin II receptor type 1; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CYP450, cytochrome P450; DPI, diphenylene iodonium; eNOS, endothelial nitric oxide synthase; FMLP, N-Formyl-Met-Leu-Phe; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; H$_2$O$_2$, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; NOS, nitric oxide synthase; NOX, NADPH oxidase; NO, nitric oxide; PGE2, prostaglandin E2; PKC, protein kinase C; PMA, para-methoxyamphetamine; PR-39, proline-arginine-rich antimicrobial peptide; ROS, reactive oxygen species; S178341, 4-dimethyl-2,3,5,6-tetraiodobenzene; SDS, sodium dodecyl sulphate; SKF 525A, 2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride; TNF-α, tumor necrosis factor-alpha; TPA, 12-O-tetradecanoylphorbol-13-acetate; VAS-2870, 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5- d) pyrimidine; XO, xanthine oxidase.
1.5.3 BH4
As mentioned in Section 1.5.1.1, an increasing body of evidence demonstrates BH4 as a key regulator of eNOS in the setting of endothelial dysfunction and a number of biological processes and pathological states (593). Substantial evidence shows that supplementation of BH4 or augment endogenous BH4 level can produce beneficial effects on cardiovascular diseases such as hypertension, peripheral arterial disease, coronary artery disease, and pulmonary arterial hypertension and so on. (438, 594). These findings suggest that regulation of BH4 level is a potential strategy to improve endothelial function and prevent or treat cardiovascular diseases.

1.5.4 Natural products
Substantial studies showed that numerous natural products have antioxidant beneficial actions in the prevention or treatment of cardiovascular diseases either by scavenging the excessive ROS and/or inhibiting ROS generating enzymes to block the ROS generation (501). Since the NOXs family is the major contributor of vascular ROS, identifying novel and specific NOX inhibitors from natural products represents a promising approach. For example, polyphenols and flavonoids, naturally occurring in a wide range of plants, are capable of reducing oxidative stress and prevention of cardiovascular diseases (501, 595, 596). It has been reported that polyphenols not only scavenge superoxide radicals but also modulate the activity and expression of NOXs (597). In agreement with previous results, Ryszawa et al. (598) showed that various polyphenols inhibited NOXs in a number of tissues including vessels and platelets. On the other hand, accumulating evidence showed that numerous natural products can also regulate NO bioavailability by targeting NOS. For example, Chung et al. (599) recently showed that syringaresinol produced vasorelaxtative effect through phosphorylation and demerization of eNOS. These findings suggest that natural products can be a promising source of therapeutic drug to treat cardiovascular diseases by alleviating oxidative stress and amelioating NO bioavailability.

Of note, Danshen is a very commonly used natural product in the treatment of cardiovascular and cerebrovascular diseases such as atherosclerosis, coronary artery
disease, stroke in the Chinese medicine setting. Substantial evidence suggests that its vascular beneficial actions are attributable to its numerous pharmacologically active components (600, 601). The vascular protective effects of *Danshen* components will be discussed in Section 1.6 and 1.7.
1.6 Overview of Danshen

Danshen is the dried root of Salvia miltiorrhiza Bunge which belongs to the family of Labiatae. It has been widely used to in Asian countries such as China and Japan, to a lesser extent, in western countries such as U.S. (600, 601). Danshen is indexed in Chinese Pharmacopoeia (2010) with more than 35 formulations and concoctions containing its water extracts, ethanol extracts or their combination (602). There are numerous dosage forms of Danshen or Danshen combination available on market including tablets, capsules, granules, injectables, oral liquids, sprays and dripping pills (600, 601). Of all dosage forms been available, Fufang Danshen Tablet and Fufang Danshen Dripping Pills are the two most widely used products (602).

1.6.1 Chemical constituents

There are numerous chemical compounds isolated and identified from the Danshen. These compounds are mainly classified into lipophilic and hydrophilic categories. Most of the lipophilic compounds are diterpene chinone compounds of tanshinone type, and so far there are about 40 diterpene quinones have been isolated and identified including tanshinone I (Tan I), tanshinone IIA (Tan IIA), tanshinone IIB (Tan IIB), cryptotanshinone (CT) and others (603). The majority of hydrophilic compounds are phenolic acids. There are over 40 phenolic acids have been isolated from aqueous extracts of Danshen (604), such as salvianolic acids, danshensu and so on. In addition to those two major groups, other compounds such as baicalin, ursolic acid and flavanone have also been found. The structural properties of some Danshen compounds are listed in (Table 1.7).
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All date were generated by ACD/PhysChem Suite.
1.6.2 Pharmacology

The capability of promoting blood circulation and removing blood stasis is the basis of medical use of Danshen for the treatment of numerous ailments. It is commonly used for the treatment of cardiovascular and cerebrovascular diseases such as coronary artery disease and stroke. The beneficial actions include improving microcirculation, vasodilating coronary artery, suppressing the formation of thromboxane, inhibiting platelet adhesion and aggregation, and protecting against myocardial ischemia (600, 601).

1.6.2.1 ADME/Tox

There is still not enough data on absorption, distribution, metabolism, extraction and toxicity (ADME/Tox) of Danshen or Danshen related products except several active components (600, 601). For example, danshensu and Tan IIA, the two major components in Danshen, exhibited a rapid absorption after oral administration of either extract formulation or individual components in both animal and human (600, 601, 605). The half-life of danshensu following sublingual administration was reported to be much longer than that after oral dosing. On the contrary, salvianolic acid B (Sal B), a major hydrophilic component, was poorly absorbed in animal studies (606). Protocatechuic aldehyde exhibited a double peak concentration after absorbed orally (607), and protocatechuic acid was a potential oxidative metabolite of protocatechuic aldehyde (605). CT was converted to Tan IIA after intravenous administration (608).

It has been reported that Danshen products may be associated with a number of clinically important herb-herb and herb-drug interactions leading to adverse outcomes (609-611). The most well-known adverse effect of Danshen is that it causes a therapeutic failure of warfarin, a highly efficacious oral anticoagulant with a narrow therapeutic index. The findings showed that Danshen could influence the pharmacokinetic and pharmacodynamic profiles of warfarin. The underlying mechanism is that tanshinones can alter the metabolism of warfarin by modulating the metabolizing enzyme CYP1A2, 2C9 and 3A4. Danshen has been shown to cause inhibition and induction of certain CYPs (612-616) as well as drug transporters such
as P-glycoprotein (P-gp) (617), thus, it is possible to affect ADME of many drugs metabolized by CYPs and transporter. For example, diazepam and midazolam exhibited a rapid metabolism due to CYP induction caused by Danshen (614, 618). In addition to herb-drug interaction, Danshen related herb-herb interactions have been reported as well. Yang et al. (619) showed that the herb-herb interactions were involved in pharmacokinetic profiles of Sal B, Tan IIA, ginsenoside Rb1, ginsenoside Rg1 and notoginsenoside R1 in the single form or the combination form. Moreover, pharmacokinetic interactions among danshensu, protocatechuic aldehyde, salvianolic acid A (Sal A) and Sal B has been also reported (620).

To ensure the clinical use of Danshen of Danshen related product, further investigations on ADME and drug interactions are needed. However, due to the multiple components and complex nature of Danshen, challenge remains.

1.6.3 Clinical application

The main clinical application of Danshen is for treating cardiovascular and cerebrovascular diseases such as coronary artery disease and stroke. It has also been used for the treatment of hyperlipidaemia and hypertension (600, 601). It has been suggested that the therapeutic effect of Danshen on angina pectoris is attributable to its properties of improving microcirculation and coronary vasodilatation, inhibiting platelet adhesion and aggregation, protecting against myocardial ischemia, and suppressing the formation of thromboxane (600, 601).

1.6.3.1 Coronary artery disease

The most important indication of Danshen is coronary artery disease including angina pectoris, coronary artery spasm, and myocardial infarction. Danshen has been widely used to treat patients with angina pectoris either alone or in combination with other conventional drugs or herbal ingredients, and the efficacy has been confirmed by systematic assessment on randomized controlled trials (621), non-inferiority trial (622), and meta-analysis (623). Moreover, it has been reported that Danshen relieved symptoms of angina as several other traditional Chinese aromatic and warm herbal medicines (624). In addition, Danshen has been shown to be effective in protecting
myocardial infarction (625). It reduced the infarct size and mortality in rats comparable to ramipril (625).

1.6.3.2 Stroke

Danshen has been indicated as a standard treatment for cerebrovascular diseases, including stroke, in China (600, 601). However systematic reviews on randomized controlled trials suggest that there is a lack of support on its efficacy in disability improvement after ischemic stroke. This might be due to the small size of clinical trials (many of these trials were not placebo controlled), or the criteria for efficacy were not clearly defined. Furthermore, the benefits of Danshen in stroke may be negated or outweighed by the high bleeding complications in stroke from its antiplatelet effect (vide supra). As such, larger size of randomized controlled trials with defined efficacy end points are warranted to evaluate the therapeutic effect of Danshen for the treatment of acute ischemic stroke.

1.6.3.3 Hyperlipidaemia

Substantial studies showed that Danshen exerted beneficial effect on regulating cholesterol, triglyceride, and LDL and high density lipoprotein (HDL) cholesterol levels. Increased plasma cholesterol levels, especially the LDL cholesterol, predispose to coronary atherosclerosis. A study of 96 elderly patients with hyperlipidaemia showed that Danshen was capable of decreasing the levels of total plasma cholesterol and LDL, and reducing the ratio of thromboxane B2 to 6-keto-prostaglandin F1α, D-dimer and fibrinogen, which in turn improved the blood coagulation system and endothelial functions (626). Xiang et al (627) also observed a greater reduction of cholesterol and triglyceride after Fufang Danshen Dripping Pill treatment compared to isosorbide dinitrate. Furthermore, there are several studies showing that total cholesterol, triglyceride, and LDL cholesterol levels were significantly reduced, and HDL cholesterol was significantly raised after the Fufang Danshen Dripping Pill treatment (600, 601).
1.6.3.4 Hypertension

Traditionally, *Danshen* has been used for treatment of hypertension in Asian countries including China, Korea and Japan (600, 601). It is suggested that the antihypertensive effect of *Danshen* is attributable to the inhibition of angiotensin converting enzyme, an essential regulatory enzyme of the renin-angiotensin system (628), and acting on eNOS (629), as well as salutary property on the rheology of erythrocytes (630). In addition, *Danshen* has been reported as a complementary therapy for pregnancy induced hypertension (631).
1.7 Effects of bioactive compounds of *Danshen* on endothelial function

There is an increasing interest in pharmacologically effective *Danshen* compounds and theirs derivatives. Recently, increasing attentions were attracted on the underlying mechanisms of actions for *Danshen* active components such as Tan I, Tan IIA, Tan IIB, CT, danshensu, Sal A/B, ursolic acid and their related compounds (600, 601).

1.7.1 Lipophilic constituents

Tanshinone, as the main lipophilic bioactive constituents (*Figure 1. 6*) isolated from *Danshen*, have been used either alone or combination form for treating cardiovascular diseases. Tan I, Tan IIA, Tan IIB, CT and dihydrotanshinones are the major tanshinones which have been extensively studied both *in vitro* and *in vivo*. The other hydrophobic constituents such as 1,2-dihydrotanshinquinone, 1,2,15,16-tetrahydrotanshiquinone, dihydroisotanshinone I, acetyltanshinone IIA, isotanshinone IIA, tanshinone VI, isocryptotanshinone, neocryptotanshinone II, oleoyl neocryptotanshinone, oleoyl danshenxinkun A, neo-tanshinlactone, tanshinlactone A, methylenetanshinquinone, methyltanshinonate, 11-hydroxymiltiodiol, 1-oxomiltirone, miltirone, ferruginol, tanshinol A, tanshinol B, danshenxinkun A, B, D. also have been studied.
Figure 1. 6. Lipophilic compounds isolated from Danshen.
Figure 1.6. Continued.
Figure 1. 6. Continued.
1.7.1.1 15, 16-Dihydrotanshinone I

It has been showed that 15,16-dihydrotanshinone I was capable of down-regulating the levels of TNF-α, IL-4, COX-2-mediated prostaglandin E2 (PGE2) and iNOS derived NO; it has also been demonstrated that 15,16-dihydrotanshinone I was able to suppress the activation of NF-κB and AP-1 and inhibit the expression of IL-1β, TNF-α, and TNF-α converting enzyme and iNOS (632-635). It has been suggested that down-regulation of pro-inflammatory cytokine and chemokine production (PGE2, TNF-α, IL-1β, IL-4 and NO) by inactivating NF-κB and AP-1 pathways and inhibition of platelet aggregation are involved in actions of 15,16-dihydrotanshinone I contributing to its cardiovascular protective effects (632-635). Moreover, Park et al. (636) showed that 15,16-Dihydrotanshinone I concentration-dependently inhibited platelet aggregation with IC50 of 8.7±5.6 µM, and suppressed intracellular calcium Ca2+ mobilization and collagen induced liberation of [3H] arachidonic acid from [3H] arachidonic acid-incorporated rabbit platelet, which indicates that the underlying mechanism of the inhibitory effect of 15,16-dihydrotanshinone I on platelet aggregation was through suppression of Ca2+ mobilization and arachidonic acid liberation.

1.7.1.2 Dihydrotanshinone

The bioactive actions of dihydrotanshinone have been reported, including regulation of Ca2+ influx and inhibition of the production of inflammatory cytokines and chemokines (635). The relaxant effect of dihydrotanshinone was studied in rat-isolated coronary artery by Lam et al. (637), which found involving suppressing the Ca2+ influx in the vascular smooth muscle without affecting endothelium, muscarinic receptors, β-adrenoceptors, adenylyl cyclase, and guanylyl cyclase involved pathways. The negative regulation of IL-12 and IFN-γ production of dihydrotanshinone has been reported with down-regulation of inflammatory cytokines and chemokines production by suppressing the promoter activation of IL-12 p40 gene and NF-κB binding to the κB site (638). These results suggest that the regulation of inflammatory response and the influx of Ca2+ are at least partially accountable for the vascular protective effects of dihydrotanshinone.
1.7.1.3 Dihydrotanshinone I

The anti-angiogenesis effect of dihydrotanshinone I was studied in vitro and in vivo by Bian et al. (639). The results showed that dihydrotanshinone I had an inhibitory effect on angiogenesis by inhibiting cell proliferation, migration, invasion and tube formation as well (639).

1.7.1.4 Tanshinone I

Tan I is one of the most abundant pharmacologically active tanshinones isolated from Danshen. A number of experimental studies have showed that Tan I can prevent or slow the progression of a wide range of diseases including cardiovascular diseases and cancer (640, 641). Nizamutdinova et al. (640) examined the effect of Tan I on the expression of ICAM-1 and VCAM-1, and found that Tan I exhibited a dose-dependent inhibitory effect on ICAM-1 and VCAM-1 expressions in HUVECs under the simulation of TNF-α for 6 hr; and an inhibitory effect on adesion of monocyte U937 to HUVECs was also observed by pretreating with Tan I which can be mimicked by RNA interference with ICAM-1 and VCAM-1 siRNA. Given adhesion molecules play a key role in endothelial function, the action of Tan I on adhesion molecules may be implicated in its anti-inflammation actions.

1.7.1.5 Tanshinone IIA

The cardiovascular effects of Tan IIA have been extensively studied both in vitro and in vivo (642). It has been demonstrated that tanshinone IIA can modulate various intracellular targets such as lowering the concentration of Ca$^{2+}$ through ATP sensitive K$^+$ channel (643), suppressing miR-1 expression through p38 MAPK signal pathway (644, 645), blocking Calcineurin/NFATc3 pathway (646), rescuing cardiac myocytes from oxidative stress induced damage and apoptosis (647), agonizing human cardiac KCNQ1/KCNE1 potassium channels (648), preventing cell proliferation (649), regulating estrogen receptor (650, 651), modulating angiogenesis (652), mediating platelet aggregation (653), suppressing monocyte chemoattractant protein-1 expression (654).
1.7.1.6 Sodium tanshinone IIA sulfonate

Sodium tanshinone IIA sulfonate is a derivative of Tan IIA and has been used in China for treating cardiovascular diseases including myocardial infarction and angina pectoris (600). The underlying mechanisms have been proposed that sodium tanshinone IIA sulfonate may prevent the generation of superoxide in endothelium (655). Recently, it has been shown that sodium tanshinone IIA sulfonate inhibited TNF-α and IFN-γ production through modulating NF-κB and IFN-γ/STAT1 signalling pathways (656). In addition, it has been reported that sodium tanshinone IIA sulfonate also down-regulated the level of Ca^{2+} in cells (657). It is believed that the beneficial effect of sodium tanshinone IIA sulfonate may act by regulating oxidative stress, inflammation response and Ca^{2+} level in cells.

1.7.1.7 Tanshinone IIB

Tan IIB, a major active ingredient of Danshen hydroxylase by CYP2A6, exhibits protective effects against ECs injury, inhibiting platelet aggregation and ameliorating the microcirculatory disturbance (658).

1.7.1.8 Tanshinone VI

Tanshinone VI is an abietane diterpene extracted from the root of *S. miltiorrhiza* Bunge. Studies have shown that tanshinone VI can protect the myocardium against ischemia induced derangements and hypoxia/reoxygenation injury (659). It also attenuated progression of *in vitro* myocardial remodeling (660).

1.7.1.9 Cryptotanshinone

CT is structurally similar to Tan IIA except C-15 position of dihydrofuran ring. It has well-documented antioxidative and anti-inflammatory effects (661, 662). CT has been shown to scavenge peroxyl radicals (663), down-regulate inflammatory cytokines and chemokines (eg TNF-α, IL-6, IL-12, IFN-γ) through inhibiting NF-κB, MAPK and AP-1 signalling pathways (635, 638, 664). It also inhibited iNOS-mediated NO production and the activation of extracellular-regulated kinase (ERK) and mobilization of NF-κB (665), down-regulated COX-2-mediated PGE2 (632),
inhibited MMP-9 production and ERK, c-Jun NH2-terminal kinase (JNK) and MAPK signalling pathways (666), decreased ET-1 expression and increases activity and expression of eNOS via NF-κB involved pathway (667), reduced the activity of COX2, and down-regulated the level of prostaglandin E2 and reactive oxygen species generation mediated by COX-2 (668). In addition, CT was showed to attenuate myocardial ischemia/reperfusion injury in vivo by reducing the infarct size and improving ischemia and reperfusion induced myocardial contractile dysfunction. In vitro experiments indicated that CT suppressed the expressions of VCAM-1 and ICAM-1 induced by TNF-α (662), reduced lactate dehydrogenase leakage, glutathione (GSH) depletion, lipid peroxidation and free radical generation (669), down-regulated the expression of ICAM-1 and VCAM-1, increased NO generation, and inhibited monocyte adhesion to HUVECs (661). These findings indicate that the mechanisms of beneficial actions of cryptotanshinone are largely due to its anti-inflammatory and anti-oxidant effects.

1.7.1.10 Other compounds

Oleoyl neocryptotanshinone and oleoyl Danshenxinkun A are two new fatty tanshinones isolated from S. miltiorrhiza Bunge with an inhibitory effect on rabbit platelet aggregation induced by arachidonic acid (670). Besides, tanshinlactone A is a new diterpenoid tanshinone compound isolated and identified from S. miltiorrhiza (671). The bioactivities of tanshinlactone A has been drawn interest from pharmacologist and life scientist. The inhibitory effect of tanshinlactone A on pro-inflammatory cytokines (IL-2 and IFN-γ) gene expression has been observed (672). These observation suggest that tanshinlactone A’s anti-inflammatory effect may partially account for its putative pharmacological activities.

1.7.2 Hydrophilic constituents

Hydrophilic constituents (Figure 1. 7) from S. miltiorrhiza are comprised of numerous active compounds. Salvianolic acids are the most abundant hydrophilic compounds extracted from Radix S. miltiorrhiza containing polyphenolic structure with antioxidation effect, to a less extent, Danshensu, ursolic acid, rosmarinic acid, caffeic acids, lithospermic acids and protocatechuic acid and other related
compounds were isolated and identified from *Danshen*. Recently, there is increasing number of studies focused on hydrophilic compounds. These compounds have been demonstrated with various activities including anti-oxidation, anti-platelet aggregation, anti-atherosclerosis and so on (601, 673-677). It is believed that the activities of the hydrophilic extract partially explain the beneficial effects of *Danshen*.
Figure 1. 7. Hydrophilic compounds isolated from Danshen.
Figure 1.7. Continued.
1.7.2.1 Salvianolic acid A

Sal A is the most potent anti-oxidant in water-soluble phenolic acids from S. miltiorrhiza. Accumulating evidence suggests that the clinical beneficial effects of Danshen on vascular disorders are in part attributable to the activities of Sal A including inhibitory effect on platelet aggregation, cell adherence, lipid peroxidation (678), in particular, the anti-oxidation effect (679, 680). Yang et al. (681) suggested that Sal A protected against vascular endothelial dysfunction by reducing oxidative stress, meanwhile, Sal A exhibited a regulatory effect on the activity and expression of eNOS in cultured cells (682). Sal A exhibited high scavenging activity on free radicals (683-685). It also was found to inhibit potently lipid peroxidation (684, 686, 687). Sal A has shown with antithrombotic and anti-platelet aggregation activities (688, 689). Jiang et al. (690) showed that Sal A also inhibited leukocyte adhesion by suppressing expression of ICAM-1 in brain micro-vascular endothelial cells. These findings suggest that Sal A has therapeutic potential in treating vascular disorders.

1.7.2.2 Salvianolic acid B

Sal B is a most abundant active phenolic acid in Danshen. Accumulating evidence suggests that Sal B possesses a broad range of activities, among these, the antioxidative stress property of Sal B is a major contributor to its cardiovascular protective actions. A number of studies have shown that Sal B exhibited a potent superoxide anion radical scavenging activity in vitro model by targeting ROS/PI3K/AKT/MEK/ERK1/2 pathway and NOX system (687, 691-697). In hypercholesterolemic animal model in rabbits, it also has been shown that Sal B inhibited endothelial damage and LDL oxidative (698). Recently, it has been reported that Sal B prevented ECs free oxidative injury by scavenging radicals (693, 699). Hung et al. (700) showed that the oxidative stress induced by homocysteine can be attenuated by Sal B. Besides, Sal B has been reported to scavenge peroxides and suppressed the expression of adhesion molecules in vascular endothelium and leukocytes, which may be related to the ameliorating effect of Sal B on microcirculation disturbance (658, 701).
In addition, the anti-inflammatory effect of Sal B may also contribute to its vascular protection. For example, Sal B exhibited anti-inflammatory effect both in vitro and in vivo by suppressing VCAM-1 and ICAM-1 expressions (TNF-α and IL-1β) (702, 703), and regulating activation of NF-κB and AP-1 DNA binding activities in vascular ECs which play a role in the development of atherosclerosis (704, 705). Besides, Sal B has been shown to modulate EC hyperpermeability which is implicated in development and progression of inflammation and subsequent ischemic reperfusion injury and atherosclerosis (706, 707). Moreover, Sal B has been demonstrated with vasodilating effect through its regulatory effect on ion channel (708, 709), platelet adhesion (710) and MMP-1, -2, and -9 activities (711).

1.7.2.3 Danshensu

Danshensu (3-(3,4-dihydroxyphenyl) lactic acid) has been extensively investigated on its vascular protective actions. It is believed that antioxidant activities and anti-inflammatory effects of danshensu contribute to the beneficial actions of Danshen. Danshensu has shown with protective effects on ECs from oxidative injury (699, 712). It reduced lipid peroxidation and the expression of IL-6 and TNF-α (713, 714); enhanced the production and activity of SOD (712, 714); increased protein level of Cx43 (712); scavenged xanthine- and xanthine oxidase-generated superoxide (715), and protected ECs from superoxide induced damage by suppressing cell apoptosis and increased the expression of CD40 (716). It seems that the free radical scavenging activity and eNOS-NO modulator properties of danshensu may be mainly responsible for its actions including those coronary arteries dilation, platelet aggregation inhibition, microcirculation improvement, and protection of myocardium from reperfusion injury of the ischemic heart, prevention of cell apoptosis (601).

Danshensu has been found with beneficial effects on homocysteine induced endothelial dysfunction by reversing decrease in cell viability and disruption of capillary-like structure formation (717), and maintaining the HUVECs integrity (706, 707). Besides, danshensu has been reported with regulation of intracellular level of Ca²⁺ and K⁺. Lam et al. (718) showed that danshensu relaxed 5HT-precontracted
coronary artery rings and abolished CaCl$_2$-induced vasoconstriction, suggesting that the inhibition of Ca$^{2+}$ influx in the vascular smooth muscle cells contributes to the vasorelaxant effect of danshensu. Danshensu may also activate K$^+$ channels (708).

1.7.2.4 Ursolic acid

The cardiovascular protective activities of ursolic acid have been showed in HUVEC (719). Ursolic acid suppressed the NOX-dependent ROS generation and inhibited the expression of NOX4, a subunit of NOX, increased eNOS expression and NO production (719). These results indicate that ursolic acid may regulate the activity and expression of NOXs and eNOS.

1.7.2.5 Salvianolate

Salvianolate has been shown to suppress the release of endothelin (720). Recent work showed that it affected cell adhesion by regulating inflammatory cytokines and chemokines. Sui et al. reported that salvianolate down-regulated the expression of IL-1$\beta$ and TNF-$\alpha$ (721).

1.7.2.6 Magnesium lithospermate B

Magnesium lithospermate B is has been shown with vascular protective activities in vitro and in vivo, including scavenging superoxide and regulating intracellular Ca$^{2+}$ (722-724). Yokozawa et al. demonstrated that magnesium lithospermate B had renal protective effect by scavenging superoxide and prevent cell injury (725, 726). Magnesium lithospermate B regulated Ca$^{2+}$ influx and NO release in ECs (724), protected vascular ECs from superoxide damage (727). Recent work showed that magnesium lithospermate B also inhibit XO derived superoxide (728). These findings indicate that magnesium lithospermate B may have a potential to treat vascular disorders.

1.7.2.7 Magnesium tanshinoate B

Magnesium tanshinoate B is an active compound isolated and purified from Danshen. A substantial attention has been received on the bioactivity of magnesium
tanshinoate B, especially, its vascular protective activity. It has been shown that magnesium tanshinoate B was able to modulate NO bioavailability by enhancing the activity and the expression of eNOS in human ECs (729). It also inhibited LDL oxidation in cultured cells (730, 731). These effects suggest that magnesium tanshinoate B may be used to treat vascular disorders associated to lipid peroxidation and NO deficiency.

1.7.2.8 Rosmarinic acid

Rosmarinic acid, a caffeic acid dimer, is one of the major phenolic constituents in *Danshen* with a broad range of bioactivities. It has been reported that rosmarinic acid had a strong anti-lipid-peroxidation activity by scavenging $O_2^-$ *in vitro* (687), and antiplatelet effects as well (732). A recent study has shown its anti-lipid peroxidation, radical scavenging, antioxidation of LDL activities (733). These findings suggest rosmarinic acid is, may contribute to the beneficial actions of *Danshen* on oxidative damage related disease.

1.7.2.9 Caffeic acid

Caffeic acid (3,4-dihydroxycinnamic acid) is one of the most common phenolic acids occurring in a broad range of plants including *S. miltiorrhiza* (733). *S. miltiorrhiza* is a rich source of caffeic acid derivatives with a wide spectrum of biological activities. The main biological activities of caffeic acid include antioxidant, anti-ischemia-reperfusion, anti-thrombosis and anti-hypertension. The anti-lipid peroxidation, radical scavenging and antioxidation of LDL may be responsible for the antioxidant effects of caffeic acid. It has been shown that caffeic acid inhibited lipid peroxidation (686), and had a free radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) (734).

1.7.2.10 Lithospermic acid

Lithospermic acid has been demonstrated with bioactivities including anti-inflammatory and hypouricemic activities (735), anti-HIV activity (736) and hormone-regulatory effects (737). Chen *et al.* suggested that lithospermic acid may act as therapeutic agent to treat atherosclerosis due to the potent inhibitory effect on
ROS generation and vascular smooth muscle cells proliferation and migration (738). Furthermore, it has been reported that lithospermic acid was capable of directly scavenging superoxide and inhibiting superoxide production by blocking XO involved the process of superoxide production (735). These observations indicate that lithospermic acid may have therapeutic potential in the prevention of oxidative stress associated diseases including atherosclerosis.

1.7.2.11 Lithospermic acid B

Lithospermic acid B is an active component isolated from *S. miltiorrhiza* with a broad range of pharmacological activities including antihypertensive effect (739), and protective actions against hepatitis and renal injury (725, 740, 741). It has been reported that lithospermic acid B had endothelium-dependent vasodilating activity (742). Recent work has shown that lithospermic acid B reduced oxidative stress by suppressing the production of malondialdehyde (MDA) via the inhibition of lipid peroxidation (743). These findings suggest that lithospermic acid B may be a new therapeutic agent to treat oxidative stress associated diseases.

1.7.2.12 Lithospermate B

The anti-oxidative stress effect of lithospermate B has been reported. Soung *et al.* showed that lithospermate B exhibited a potent peroxynitrite scavenging activity (744). The study also showed that the determinants of the peroxynitrite scavenging activity are the dihydroxyl group and the double bond in structure (744).

1.7.2.13 Dimethyl lithospermate

Dimethyl lithospermate is bioactive component isolated from *S. miltiorrhiza*. Kim *et al.* reported that dimethyl lithospermate was capable of reducing oxidative stress by scavenging peroxynitrite (745), which may be involved in the beneficial actions of *Danshen*. 
1.7.2.14 Protocatechuic aldehyde

Protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) is an active water-soluble component from *Danshen*. It has been shown that protocatechuic aldehyde exhibited a potent anti-oxidation activity and anti-inflammatory effects in cultured vascular ECs (746). Zhou *et al.* showed that protocatechuic aldehyde was capable of targeting gene and protein expressions of VCAM-1 and ICAM-1 and down-regulating the release of those two vascular adhesion molecules in ECs (746). Furthermore, protocatechuic aldehyde suppressed the activation of NF-κB and AP-1 DNA binding activities (746). These findings suggest that protocatechuic aldehyde may be attributable to the anti-oxidation activity and anti-inflammatory activity of *Danshen* through targeting NF-κB and AP-1 involved signalling pathway.

1.7.2.15 Protocatechuic acid

Protocatechuic acid, a structurally typical active phenolic acid compound, has been reported with capability of scavenging of oxygen free radicals and protecting vascular ECs from homocysteine induced endothelial dysfunction (717, 747).

1.7.2.16 Ailanthoidol

Ailanthoidol (3-deformylated 2-arylbenzo[b]furan) is a neoligan from *S. miltiorrhiza* or *Zanthoxylum ailanthoides* Bunge and is used in Chinese traditional herbal medicine. Lee *et al.* reported that ailanthoidol had a radical quenching property and an inhibitory effect of ailanthoidol on TPA induced oxidative stress and anti-inflammatory effects as well (748). Recently, Kim *et al.* observed that ailanthoidol could strongly suppress the production of inflammatory cytokines and chemokines including IL-1β, IL-6, TNF-α, iNOS, and COX-2 through inhibiting NF-κB activation (749). These findings indicate ailanthoidol may be a potential resource for treating oxidative stress and inflammatory associated diseases.

1.7.2.17 3,4-Dihydroxyphenyl lactic acid

Like the effects of other hydrophilic compounds, the pharmacological activities of 3,4-dihydroxyphenyl lactic acid have been investigated both *in vitro* and *in vivo*
It has been shown as a superoxide anion free radicals scavenger removing superoxide generated from the reaction system of xanthine and xanthine oxidase and also protects the mitochondrial membrane from the ischemia-reperfusion injury and lipid peroxidation (715). 3,4-dihydroxyphenyl lactic acid was capable of reducing the expression of adhesion molecules CH11b and CD18 as well as the production of TNF-α, superoxide anion and hydrogen peroxide by neutrophils induced by LPS (701, 750, 751). Recently, 3,4-dihydroxyphenyl lactic acid has been reported with a significant inhibitory effect on polymorphonuclear cell infiltration, IL-6 production, IκB-α degradation and the nuclear translocation of NF-κB p65 subunit protein. It has been found that 3,4-dihydroxyl group may play an important role as modification or depletion of dihydroxyl group eliminated its peroxide scavenging potential (734).

**1.7.2.18  2,10,11-trihydroxy-8-methoxy-1,6,7,8-tetrahydro-2H-benzo[e]azecine-3,5-dione**

2,10,11-trihydroxy-8-methoxy-1,6,7,8-tetrahydro-2H-benzo[e]azecine-3,5-dione is a new cyclic phenyllactamide, namely salviamiltamide, isolated from the rhizome of *S. miltiorrhiza*. It has been shown that 2,10,11-trihydroxy-8-methoxy-1,6,7,8-tetrahydro-2H-benzo[e]azecine-3,5-dione had radical scavenging activity on DPPH with a comparable IC50 value to that of L-ascorbic acid (752).

**1.7.2.19  2-Allyl-3,4-dihydroxybenzaldehyde**

2-Allyl-3,4-dihydroxybenzaldehyde (S-3-1) is a salvianolic acid A derived synthetic derivative with various bioactivities (753-755). It has been reported that S-3-1 possesses an effective antioxidant activities. S-3-1 exhibited a superoxide scavenging activities on DPPH, superoxide anion and hydroxyl radicals (753). Moreover, S-3-1 had inhibitory effect on lipid peroxidation in microsome fraction from rat liver induced by FeSO4 and cysteine (753).

**1.7.2.20  (+)-1-Hydroxypinoresinol-1-O-β-D-glucoside**

(+)-1-hydroxypinoresinol-1-O-β-D-glucoside is a furanofuranoid lignan glycoside isolated from the rhizome of *S. miltiorrhiza*. It has been reported that (+)-1-
hydroxypinoresinol-1-O-β-D-glucoside had superoxide scavenging activity. Kang et al. showed that (+)-1-hydroxypinoresinol-1-O-β-D-glucoside scavenged peroxynitrite, total ROS and DPPH radical with IC50 values of 3.23 ± 0.04, 2.26 ± 0.07 and 32.3 ± 0.13 µM, respectively (756). These radical scavenging activities were comparable to that of L-ascorbic acid.

1.7.2.21 2-(3’-Methoxy-4’-hydroxy-phenyl)-5-(3-hydroxypropyl)-7-methoxybenzo[b]furan-3-carbaldehyde

2-(3’-Methoxy-4’-hydroxy-phenyl)-5-(3-hydroxypropyl)-7-methoxy-benzo[b]furan-3-carbaldehyde (XH-14) is bioactive ingredient isolated from *S. miltiorrhiza*, being a member of benzo[b]furan lignan family. The lignans have been reported with a broad spectrum of biological activities including antiplatelet (757), antioxidant (758, 759), anti-inflammatory (760). XH-14 has been known as a relatively potent adenosine antagonist proposed to treat ischaemic bradyarrhymias, cardiac arrest and renal disease with an advantage of relatively high water solubility and binding affinity and good bioavailability (761). Recently, Sung et al. reported that three novel XH-14 derived benzo[b]furan derivatives inhibited inflammatory responses and may have a potential to treat obesity-associated inflammatory and metabolic diseases (762).

1.7.2.22 Isopropyl-β-(3,4-dihydroxyphenyl)-α-hydroxypropanoate

Isopropyl-b-(3,4-dihydroxyphenyl)-a-hydroxypropanoate is a new metabolite of *Danshen* found in rat brain, designated ND-309 (763). It has been shown that ND-309 reduced the production of reactive oxygen species, down-regulated the content of MDA, increased the activity of SOD, glutathione-peroxidase (GSH-Px) in middle cerebral artery occlusion (MCAO) rat model (763). Moreover, ND-309 increased the brain ATP content and improve mitochondrial energy metabolism (763). Thus, it may be potentially useful in developing new treatment for cerebral ischemia injury.
1.7.2.23  (2E)-2-{6-{((E)-2-carboxylvinyl}-2,3-dihydroxyphenyl}-3-(3,4-dihydroxyphenyl)propenoic acid

(2E)-2-{6-{((E)-2-carboxylvinyl}-2,3-dihydroxyphenyl}-3-(3,4-dihydroxyphenyl) propenoic acid is a novel compound designated SMND-309 and has a brain distribution (764). SMND-309 is a new derivate of salvianolic acid B which is the most abundant salvianolic acids possessing many beneficial action including reducing lipid peroxides and scavenging free radicals (678). It has been shown that SMND-309 decreased mitochondria production of reactive oxygen species and MDA and enhanced activities of mitochondrial respiratory chain complex, SOD and GSH-Px in MCAO rat model (764). These results indicate that SMND-309 may be used for treating stroke.

1.7.2.24  Isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate

Isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate (IDHP) is one of the major bioactive metabolites of Danshen with vasodilating acivity (765). Wang et al. reported that IDHP showed concentration-dependent vasorelaxant effect through inhibition of Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx via voltage-dependent calcium channels and receptor-operated calcium channels, and positive regulation of vascular tetraethylammonium-sensitive K\(^+\) channels.
1.8 Computational approaches in evaluation of chemical characteristics and ADME/Tox

Recently, the use of natural products, including herbal medicine, has been becoming increasingly popular worldwide for improving body function or manage numerous ailments, despite, in many cases, the lack of evidence for efficacy or long term toxicological data. This is not surprising as products have been recognised as sources for new drugs. However, due to the complex nature of natural product, the paucity of experimental information, and limited applicability and availability of experimental tools in ADME/Tox analysis, the natural product derived new drug development seems to be hampered in recent times.

The increasing use of natural products in the general population in recent years highlights the need for proper safety assessment of these products as many of these products do not need rigorous toxicology tests before they enter into market (766). The lack of rigorous toxicology tests of these products may be associated with increasing number of relevant adverse events reported for these products including herbal toxicity. Such as Callilepis laureola induced acute kidney injury, convulsions, abdominal pain, diarrhea and vomiting (767-769), herbal extracts or preparations of cat’s claw (770) and propolis (771) caused acute renal failure, and Ephedra sinica (Ma huang) with hepatoxic effect (772, 773). Thus, the safety issue of natural products has been raised.

One of the main reasons hindering many early drug candidates reaching market is the unfavourable ADME properties and drug induced toxicity. From a commercial perspective, fast and reliable in vitro assessment strategies are needed to filter out problematic molecular at the earliest stages of discovery rather than during the more costly drug development phases. As a consequence, over the past decade, ADME/Tox screening studies have been incorporated earlier in the drug discovery phase. Predicting biological fate and toxicological properties of compound has been a critical step in early stage of drug development process to avoid late stage attrition with huge cost (774-776). It has been widely accepted that not only the efficacy but also the ADME/Tox profiles of drug candidates are of importance in optimizing a
chemical compound. There have been a number of drugs withdrawn from market due to at least partly poor ADME/Tox properties (Table 1.8), including nomifensine, temafloxacin, flosequinan, mibefradil, rezulin, and rofecoxib. It is therefore of importance to evaluate both efficacy and ADME and toxicological properties of natural compounds.
<table>
<thead>
<tr>
<th>Drug name</th>
<th>Year of withdrawal</th>
<th>Reason for withdrawn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drotrecogin alfa (Xigris)</td>
<td>2011</td>
<td>Lack of efficacy</td>
</tr>
<tr>
<td>Rosiglitazone (Avandia)</td>
<td>2010</td>
<td>Risk of heart attacks and death (withdrawn in Europe)</td>
</tr>
<tr>
<td>Gemtuzumab ozogamicin (Mylotarg)</td>
<td>2010</td>
<td>Risks of veno-occlusive disease (withdrawn in the U.S. due to increased)</td>
</tr>
<tr>
<td>Sibutramine (Reductil/Meridia)</td>
<td>2010</td>
<td>Risk of cardiovascular disease (withdrawn in Europe, Australasia, Canada, and the U.S.)</td>
</tr>
<tr>
<td>Efalizumab (Raptiva)</td>
<td>2009</td>
<td>Risk of progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>Rimonabant (Acomplia)</td>
<td>2008</td>
<td>Risk of severe depression and suicide</td>
</tr>
<tr>
<td>Lumiracoxib (Prexige)</td>
<td>2007–2008</td>
<td>Liver damage</td>
</tr>
<tr>
<td>Pergolide (Permax)</td>
<td>2007</td>
<td>Risk of heart valve damage (withdrawn in the U.S.)</td>
</tr>
<tr>
<td>Tegaserod (Zelnorm)</td>
<td>2008</td>
<td>Heart attack and stroke</td>
</tr>
<tr>
<td>Aprotinin (Trasylol)</td>
<td>2008</td>
<td>Risk of complications or death</td>
</tr>
<tr>
<td>Inhaled insulin (Exubera)</td>
<td>2007</td>
<td>Long-term safety issue and high cost (withdrawn U.K.)</td>
</tr>
<tr>
<td>Ximelagatran (Exanta)</td>
<td>2006</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Thioridazine (Melleril)</td>
<td>2005</td>
<td>Cardiotoxicity (withdrawn in U.K.)</td>
</tr>
<tr>
<td>Pemoline (Cylert)</td>
<td>2005</td>
<td>Hepatotoxicity (withdrawn in U.S.)</td>
</tr>
<tr>
<td>hydromorphone extended-release (Palladone)</td>
<td>2005</td>
<td>Overdose dangers when administered with alcohol</td>
</tr>
<tr>
<td>mixed amphetamine salts (Adderall XR)</td>
<td>2005</td>
<td>Risk of stroke (withdrawn in Canada)</td>
</tr>
<tr>
<td>Co-proxamol (Distalgesic)</td>
<td>2004</td>
<td>Overdose dangers (Withdrawn in U.K.)</td>
</tr>
<tr>
<td>Drug</td>
<td>Year</td>
<td>Risk</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>Rofecoxib (Vioxx)</td>
<td>2004</td>
<td>Risk of myocardial infarction</td>
</tr>
<tr>
<td>Trovafoxacin (Trovan)</td>
<td>2001</td>
<td>Risk of liver failure</td>
</tr>
<tr>
<td>Cerivastatin (Baycol, Lipobay)</td>
<td>2001</td>
<td>Risk of rhabdomyolysis</td>
</tr>
<tr>
<td>Rapacuronium (Raplon)</td>
<td>2001</td>
<td>Risk of fatal bronchospasm</td>
</tr>
<tr>
<td>Cisapride (Propulsid)</td>
<td>2000</td>
<td>Risk of cardiac arrhythmias</td>
</tr>
<tr>
<td>Troglitazone (Rezulin)</td>
<td>2000</td>
<td>Risk of hepatotoxicity</td>
</tr>
<tr>
<td>Phenylpropanolamine (Propagest, Dexatrim)</td>
<td>2000</td>
<td>Risk of stroke</td>
</tr>
<tr>
<td>Amineptine (Survector)</td>
<td>2000</td>
<td>Hepatotoxicity, dermatological side effects, and abuse potential.</td>
</tr>
<tr>
<td>Temazepam (Restoril, Euhynpos, Normison, Remestan, Tenox, Norkotral)</td>
<td>1999</td>
<td>Diversion, abuse, and a relatively high rate of overdose deaths (withdrawn in Sweden and Norway)</td>
</tr>
<tr>
<td>Grepafloxacin (Raxar)</td>
<td>1999</td>
<td>Prolonged QT interval</td>
</tr>
<tr>
<td>Astemizole (Hismanal)</td>
<td>1999</td>
<td>Arrhythmias because of drug interactions</td>
</tr>
<tr>
<td>Levamisole (Ergamisol)</td>
<td>1999</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Terfenadine (Seldane, Triludan)</td>
<td>1998</td>
<td>Risk of cardiac arrhythmias</td>
</tr>
<tr>
<td>Tolcapone (Tasmar)</td>
<td>1998</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Mibefradil (Posicor)</td>
<td>1998</td>
<td>Dangerous drug interactions</td>
</tr>
<tr>
<td>Tolrestat (Alredase)</td>
<td>1997</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Chlormezanone (Trancopal)</td>
<td>1996</td>
<td>Toxic epidermal necrolysis</td>
</tr>
<tr>
<td>Alpidem (Ananxyl)</td>
<td>1996</td>
<td>Hepatotoxicity</td>
</tr>
</tbody>
</table>
Table 1.8. Continued.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Year</th>
<th>Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flosequinan (Manoplax)</td>
<td>1993</td>
<td>Risk of hospitalization or death (Withdrawn in U.S.)</td>
</tr>
<tr>
<td>Temafloxacin</td>
<td>1992</td>
<td>Allergic reactions and cases of hemolytic anemia (Withdrawn in U.S.)</td>
</tr>
<tr>
<td>Terodiline (Micturin)</td>
<td>1991</td>
<td>Prolonged QT interval</td>
</tr>
<tr>
<td>Triazolam</td>
<td>1991</td>
<td>Risk of psychiatric adverse drug reactions (withdrawn in U.K.)</td>
</tr>
<tr>
<td>Etretinate</td>
<td>1990s</td>
<td>Risk of birth defects and narrow therapeutic index</td>
</tr>
<tr>
<td>Nomifensine (Merital)</td>
<td>1986</td>
<td>Risk of hemolytic anemia</td>
</tr>
<tr>
<td>Methaqualone</td>
<td>1984</td>
<td>Risk of addiction and overdose</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>1983</td>
<td>Risk of cancer and kidney disease</td>
</tr>
<tr>
<td>Zimelidine</td>
<td>1983</td>
<td>Risk of Guillain-Barré syndrome</td>
</tr>
<tr>
<td>Ticrynafen</td>
<td>1982</td>
<td>Risk of hepatitis</td>
</tr>
<tr>
<td>Phenformin and Buformin</td>
<td>1978</td>
<td>Risk of lactic acidosis</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>1970s</td>
<td>Risk of teratogenicity</td>
</tr>
<tr>
<td>Lysergic acid diethylamide</td>
<td>1950s–1960s</td>
<td>Drug abuse</td>
</tr>
</tbody>
</table>
Chemical characteristics of a molecule determine the drug’s behaviour in pharmacokinetic, pharmacodynamic and toxicity aspects (Figure 1. 8). The structural properties of compounds, including molecular weight, H-bonds, lipophilicity, polar surface area (PSA), ionization constant (pKa), shape and reactivity, interact with the physicochemical environment affecting physicochemical properties, including solubility, permeability and chemical stability. When these structural properties interact with proteins, they affect biochemical properties, including metabolism, transporter affinity, binding and target affinity. At the highest level, when the physicochemical and biochemical properties interact with living systems they cause pharmacokinetics (PK), pharmacodynamics (PD), and toxicity, including clearance, half-life, bioavailability and LD50. By modifying the structure we may be able to control the PK, PD and toxicity properties of the compounds.
Figure 1. Chemical characteristics determine the profiles of pharmacokinetics, pharmacodynamics and toxicity.
There are a number of chemical descriptors in evaluation the profile of ADME/Tox (Table 1. 9). The most important physicochemical properties include pKa, lipophilicity (logP/D), solubility (logS), and permeability. pKa is a useful physicochemical property accounting for thermodynamics, which modulates the charge state and eventually solubility and other key properties of drug candidate. It is helpful for understanding binding of drug candidate to targets. LogP/D is a determinant to quantitatively describe a drug candidate’ ability to partition in the oily phase or biological membranes. It is also frequently utilized in explaining behaviour of some compounds in the ADME/Tox assays. Lipophilicity greatly affects solubility, permeability, and the other drug properties. Being a critical parameter affecting both in vitro and in vivo attributes, solubility measures the ability of drug candidates to dissolve and remain in aqueous or other physiological media.
<table>
<thead>
<tr>
<th>Chemical property</th>
<th>Description</th>
<th>ADME relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LogP</td>
<td>Water-octanol partition coefficient. The ratio of the respective concentrations of a compound in the octanol and water phases of a 2-phase system at equilibrium. Measures lipophilicity.</td>
<td>Oral bioavailability; BBB partitioning; intestinal absorption; plasma protein binding</td>
</tr>
<tr>
<td>LogS</td>
<td>Aqueous or water solubility usually expressed as mol/L or mg/mL</td>
<td>Oral bioavailability; BBB partitioning; plasma protein binding</td>
</tr>
<tr>
<td>LogD</td>
<td>The apparent water-octanol partition (or distribution) coefficient measured for ionic species. A combination of logP and pKa</td>
<td>Permeability coefficient; volume of distribution</td>
</tr>
<tr>
<td>pKa</td>
<td>The negative log of the acid ionisation constant. It describes the ability of an ionisable group of an organic compound to donate a proton in an aqueous medium</td>
<td>Water solubility; volume of distribution</td>
</tr>
<tr>
<td>MW</td>
<td>The sum of the atomic weights of all the atoms in a molecule</td>
<td>Oral bioavailability; BBB partitioning; CYP interactions</td>
</tr>
<tr>
<td>PSA</td>
<td>The surface area of N, O, P and S atoms</td>
<td>Oral bioavailability; intestinal absorption; BBB partitioning</td>
</tr>
<tr>
<td>No. of H-bond donors</td>
<td>Number of relatively electronegative atoms such as N, O, S or F with attached hydrogen atoms</td>
<td>Oral bioavailability; BBB partitioning; intestinal absorption</td>
</tr>
<tr>
<td>No. of H-bond acceptors</td>
<td>Number of relatively electronegative atoms such as N, O, S or halogens with an available lone pair</td>
<td>Oral bioavailability; BBB partitioning</td>
</tr>
<tr>
<td>No. of rotatable bonds</td>
<td>Number of sp3 (single) bonds, not in a ring, bound to a non-terminal heavy atom</td>
<td>Oral bioavailability; BBB partitioning</td>
</tr>
<tr>
<td>Molar refractivity</td>
<td>Molar refractivity is a measure of the volume occupied by an atom or group. It varies with temperature, index of refraction and pressure</td>
<td>Oral bioavailability; intestinal absorption</td>
</tr>
<tr>
<td>Molecular volume</td>
<td>Volume occupied by 1 mole of molecule; it equals the molecular weight divided by the density</td>
<td>CYP interactions; water solubility; intestinal absorption; oral bioavailability; BBB partitioning</td>
</tr>
<tr>
<td>Dipole moment</td>
<td>A measure of the electrical polarity of a molecule with partially charged atoms</td>
<td>Water solubility, CYP interactions; serum protein binding</td>
</tr>
</tbody>
</table>
### Table 1. 9. Continued.

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
<th>BBB partitioning; permeability coefficient</th>
<th>CYP interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free water solvation energy</td>
<td>Free energy of dissolving a compound in water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radius of gyration</td>
<td>Distance between the axis of a rotating body and its centre of gyration. A combined measure of molecular volume and shape</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscellaneous topological descriptors</td>
<td>These features describe the bond connectivity, bond types and overall shape for a given molecule</td>
<td>Water solubility; oral absorption; serum protein binding</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Wishart et al. (777).

**Abbreviation:** BBB, blood-brain barrier; CYP, cytochrome P450; F, fluoride; H-bond, hydrogen bond; N, nitrogen; O, oxygen; P, phosphate; pKa, ionisation constant; PSA, polar surface area; S, sulphate; WM, molecular weight.
These key physicochemical parameters are critical for the predictability of drug ADME/Tox properties such as absorption, cell penetration, access to the brain, volume of distribution, plasma protein binding, metabolism, and toxicity, as well as biopharmaceutical behaviour. Ionization constant is a critical physicochemical parameter on thermodynamic property to manipulate the charge state. Consequently, it affects solubility, permeability, and other key properties of drug candidate. Lipophilicity is the key determinant factor in many ADME processes including absorption and distribution and is also a factor influencing the interaction with drug metabolizing enzymes and transporters. Solubility and permeability drive the oral absorption and bioavailability.

Due to the high attrition rate from leads to drug candidate, it is necessary to assess and optimize the comprehensive drug physicochemical properties. The challenge in optimization is that the physicochemical properties of a molecule are all strongly interlinked. To date, there are numerous studies on analyzing and manipulating physicochemical properties of a molecule, pKa, log P/D, logS, and permeability (778, 779). The advance in in silico approaches in last decades has brought significant changes in drug discovery and development process, in particular, in studying ADME/Tox properties in early drug discovery and development stage and avoids late-stage expensive drug attrition (774, 780). Compared to traditional cell- or animal-based experimental procedures, which are particularly expensive, lengthy, uninformative, or offensive ones, in silico methods provide a rapid and inexpensive option to study the biological fate or properties of new chemical entity or drug candidates by using powerful computational approaches. Due to the complex nature, and high risk and cost in drug discovery and development, proper predication of the biological fate and toxicity of a new chemical entity or drug candidate in the early stage of drug discovery process will be important in improving the success rate of drug discovery. However, in some cases, these profiles are often difficult to be ascertained due to the complex process of ADME and toxic features of new chemical entity (781-783).
There are recognised difficulties in conventional drug discovery and development including the large number of compounds for in vivo studies; the lack of reliable high-throughput in vitro assays; the inability of in vitro and animal models to predict the toxicities for the large number of compounds. Thus, in silico approaches are highly valuable in studying the ADME/Tox properties of compounds in early stage of drug development including various metabolic events with the participation of Phase I and/or Phase II enzymes (e.g. metabolic stability or microsomal stability, biotransformations, prediction of metabolites and sites of metabolism, and determination of rate of metabolic reaction and ligand regioselectivity), binding to specific metabolic enzymes or drug transporters (prediction of ligand-enzyme interactions, calculation of binding energy, discrimination inhibitor versus substrate and identification of mechanism-based inhibition), and alteration on metabolizing enzymes, drug transporters, or nuclear receptors (784-791).
1.9 Aim of thesis

Endothelial dysfunction has been implicated in the pathogens of vascular diseases. eNOS uncoupling, characterized as reduced NO generation and increased superoxide production by eNOS, is a mechanism of endothelial dysfunction with a disturbance of vascular homeostasis. *Danshen*, a versatile natural product with a wide spectrum of activities, is mainly used to treat vascular diseases including cardiovascular and cerebrovascular diseases. The vascular protective effects of *Danshen* is attributable to beneficial actions of its numerous pharmacologically active components.

However, the physicochemical properties of numerous *Danshen* compounds have not been evaluated. These properties are accounting for the ADME/Tox profile of *Danshen* compounds and are determinant factors responsible for the varying pharmacological activities, and the relationship between chemical characteristics and the drug potential of *Danshen* compounds. Thus, it is necessary to analyse the physicochemical properties of *Danshen* compounds. Additionally, the increasing application of natural products for various conditions rises safety concerns associated with natural products. Thus, it needs to evaluate the biological fate, toxicity and drug interaction of natural products.

In addition, the effect of *Danshen* components including Tan I, Tan IIA and CT on eNOS uncoupling have not been previously studies. Thus, it is necessary to investigate in greater detail the effect of active *Danshen* compounds on eNOS uncoupling and the underlying molecular mechanism.

Therefore, the specific aims of this thesis are:

1) To analyse the physicochemical properties of *Danshen* compounds. We will evaluate the physicochemical parameters including most important physicochemical properties Based on physicochemical parameters; we will assess potential toxicity and predict drug-likeness of *Danshen* components.

2) To evaluate biological fate, toxicity and drug interaction of natural products by *in silico* approaches. Particularly, we will focus on drug metabolizing enzymes, transporters and nuclear receptors, including CYP450, uridine diphosphate glucuronosyltransferases (UGTs), P-gp, organic cation
transporters (OCTs), constitutive androstane receptor (CAR) and pregnane X receptor (PXR).

3) To set up an in vitro cell based model of high glucose induced eNOS uncoupling in EA.hy926 cells. In this part, we will employ biochemical approaches to examine experimental hyperglycaemia condition on cellular productions of superoxide and NO; test the expression of NOX4 and HSP90 and the ratio of BH4 to BH2.

4) To examine the effects of Tan I, Tan IIA and CT on high glucose induced eNOS uncoupling in EA.hy926 cell line, including their effects on superoxide and NO production, eNOS expression, BH4 and BH2 levels, the expressions of NOX4 and HSP90.

5) To examine the effects of new tanshinone derivatives on high glucose induced eNOS uncoupling in EA.hy926 cell line by assessing the effect on superoxide and NO production, the ratio of eNOS dimer to monomer and BH4 to BH2, the expressions of NOX4 and HSP90.
Chapter 2. Methodology

2.1 Materials and Methods

2.1.1 Chemicals and reagents
All cell culture reagents were purchased from sigma (St. Louis, MO). Primary antibodies for PI3K p110β subunit (sc-100407), HSP90 (sc-69703), β-actin (sc-47778), GTPCH1 (sc-271482) and DHFR (sc-74594) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); NOX4 antibody (ab109225) was purchased from Abcam (Cambridge, UK); eNOS antibody (SAB4300435), protease inhibitor cocktail (P8340), diphenylene iodonium (DPI) and dimethyl sulfoxide (DMSO) were obtained from sigma (St. Louis, MO). MnTBAP, DAF2-DA, L-arginine hydrochloride and N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) were purchased from Cayman Chemical (Ann Arbor, MI). Tan I, Tan IIA and CT was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). AC1 and AC2 were synthesized by department of Chemistry, RMIT University. All tested Danshen compounds were dissolved initially in DMSO and subsequently diluted to the desired experimental concentrations with culture media.

2.1.2 Computational approach
To carry out the in silico study, we retrieved the structure of Danshen compounds from two databases: PubChem Compound (http://www.ncbi.nlm.nih.gov/pccompound/) and ChemSpider (http://www.chemspider.com/). To draw the 2D structure, ChemBiodraw Ultra 12.0 was used. To analyse the physicochemical properties of Danshen compounds, the softwares: OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/), ALOGPS (http://www.vcclab.org/lab/alogps/) and ACD/ChemSketch (http://www.acdlabs.com/products/draw_nom/draw/chemsketch/) were used. To assess the toxicity and drug potential, OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/) was employed.

2.1.3 Computational approach work-flow
To conduct the computational evaluation of Danshen compounds, we follow the work flow as in (Figure 2. 1). Briefly, literature review was conducted and a
chemical library of *Danshen* compounds was established. Then, the structural and physicochemical properties of *Danshen* compounds were analysed, followed by assessing drug-like and nondrug-like properties according to Rule of 5 (Ro5) (792). If a compound violated Ro5, then it was categorized into non drug-like group; if not, it was classified into drug-like group. Finally, the potential toxicity risks and drug potential were analysed.
Figure 2.1. Work flow for computational study to evaluate pharmacological effect of Danshen compounds.
2.1.4 Drug-likeness value

Drug-likeness value was evaluated by OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/) according to the following Equation 2.1.

\[
d = \frac{\sum v_i}{\sqrt{n}}
\]

This approach was based on a list of about 5300 distinct substructure fragments with associated drug-likeness scores. The fragment list was created by shredding 3300 traded drugs as well as 15000 commercially available chemicals (Fluka) yielding a complete list of all available fragments (http://www.organic-chemistry.org/prog/peo/).

2.1.5 Drug score

The drug score of Danshen compounds was calculated by multiplying contributions of the individual properties with the Equation 2.2. It combined with drug-likeness, clogP, clogS, molecular weight and toxicity risks. ds was the drug score. \( s_i \) was the contributions calculated directly from of clogP, clogS, molweight and drug-likeness (\( p_i \)) via Equation 2.3, which describes a spline curve. Parameters \( a \) and \( b \) were (1, -5), (1, 5), (0.012, -6) and (1, 0) for clogP, clogS, molweight and drug-likeness, respectively. \( t_i \) was the contributions taken from the 4 toxicity risk types. The \( t_i \) values were 1.0, 0.8 and 0.6 for no risk, medium risk and high risk, respectively (http://www.organic-chemistry.org/prog/peo/).

\[
ds = \pi \left( \frac{1}{2} + \frac{1}{2} s_i \right) \cdot \pi t_i
\]
2.1.6 Cell culture
The HUVEC cell line EA.hy926 was kindly provided by Dr. Shanhong Ling (793, 794), Monash University Central and Eastern Clinical School, Melbourne, Victoria, Australia. The EA.hy926 cell line was previously established in the pathology department, University of North Carolina (Chapel Hill, NC, USA). The cells were cultured in low glucose (5.5 mM) or high glucose (35 mM) Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 0.5% streptomycin/penicillin and HAT media supplement (100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine) in 5% CO₂ humidity atmosphere at 37°C. Cells at passage 29-35 were used in the study.

2.1.7 MTT assay
MTT assay was used to test the viability of EA.hy926 cell line, which was performed mainly according to the method of Mosmann (795) with some modifications. All the experiments were repeated five times with four parallel wells. The spectrophotometer (BMG Labtech) was calibrated to zero absorbance using culture without cells. The relative cell viability (%) related to control wells containing cell culture media without Danshen compounds was calculated by [A]test/[A]control×100.

2.1.8 Cell treatment
Tan I, Tan IIA, CT, AC1 and AC2 were dissolved into DMSO with a stock concentration of 1.0 mM, and were freshly diluted to the desired concentration (1, 3, and 10 µM) with culture media. The final concentration of DMSO in the five Danshen compounds treated cells was at 0.08% (v/v). The control cells received the
same amount of DMSO treatment without Danshen compounds. For experiments, cells were cultured for 4-6 hr in reduced serum media (4%) prior to treatment in 6-well or 12-well plates at a density of 4x105 cells/well. Cells were treated with Danshen compounds for 24 hr after culturing in 35 mM high glucose for 24 hr without specific indication (Figure 2.2).
**Figure 2.2. Cell treatment.**

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
<th>Medium Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour</td>
<td>Seed EA.hy926 cells</td>
<td>5.5 mM D-glucose + 10% FBS + 0.5% streptomycin/penicillin + HAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hour</td>
<td>4% FBS media</td>
<td>35 mM D-glucose + 10% FBS + 0.5% streptomycin/penicillin + HAT</td>
</tr>
<tr>
<td>28 hour</td>
<td>Treat cells with Danshen Compounds</td>
<td>35 mM D-glucose + 10% FBS + 0.5% streptomycin/penicillin + HAT</td>
</tr>
<tr>
<td>52 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76 hour</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.9 Detection of NOXs-dependent superoxide production

NOXs-dependent superoxide production produced by ECs was measured by the lucigenin (5 µM) enhanced chemiluminescence assay (796, 797). In brief, cells were cultured in reduced serum media for 4 hr after seeding in 12-well plates for 24 hr, and then treated with high glucose for 24 hr initially, followed by the treatment with *Danshen* compounds for another 24 hr under high glucose condition. Cell samples, prepared by homogenizing with a freezing-thawing method in Kreps-HEPES buffer, were transferred into 96-well OptiPlate. NADPH (100 µM) and lucigenin (5 µM) were added after the background reading. Luminescence was then measured every 15s along an interval of 45min using a plate reader luminometer (BMG Labtech).

2.1.10 NO measurement

Cells were washed with Krebs Ringer Buffer (120 mM NaCl, 4.8 mM KCl, 0.54 mM CaCl2, Mg2SO4•7H2O, 11 mM glucose, 15.9 mM NaH2PO4, pH 7.2) and pre-incubated with L-arginine (100 µM in KRP, 5 min, 37°C) (798, 799). In some experiments, L-NAME (100 µM) was added 5min before the addition of L-arginine. Subsequently, 1 µM DAF-2DA was added and cells were incubated in the dark (37°C for 5 min). Then the fluorescence was measured at room temperature using a spectrofluorimeter with excitation wavelength set at 495 nm and emission wavelength at 515 nm (BMG Labtech). The band width was 10 nm for both excitation and emission.

2.1.11 Western blot analysis

Cells were washed with pre-cold PBS, lysed with lysis buffer [50 mmol Heps (pH 7.5), 150 mmol NaCl, 10% Glycerol, 1.5 mmol MgCl2 , 1% Triton x 100, 1 mmol EDTA (pH 8.0), 10 mmol NaPi, 10 mmol NaF and protease inhibitor cocktail] and centrifuged for 10 min at 13,000 rpm at 4°C (800). Equal amounts of cellular proteins were resolved by SDS-PAGE sample loading buffer (4×+2% mercaptoethanol). Samples were electrophoresed on 12% SDS-PAGE mini gels after thermal denaturation for 5 min at 95°C, and were transferred to nitrocellulose membranes. Membranes were probed with targeted primary antibodies and blotted with respective secondary antibodies. Low temperature western blot assay was used to examine the ratio of eNOS dimer and monomer (53). Protein samples without
thermal denaturation were electrophoresed on 6% SDS-PAGE mini gels and transferred to nitrocellulose membranes. Membranes were probed with anti-eNOS primary antibody and blotted with respective secondary antibody. Proteins levels were normalized to the matching densitometric values of internal control.

2.1.12 Measurement of intracellular levels of biopterins

The intracellular biopterin levels (oxidized and reduced forms) were determined by HPLC as previously described (431, 801, 802)[13,45,58]. Briefly, ECs were lysed and suspended in 12-well plates with cold extract buffer (50 mM Tris-HCl, pH7.4; 1 mM DTT; 1mM EDTA; containing 0.1 µM neopterin). Protein concentration was measured using BCA protein assay. Proteins were removed by adding 10 µl of a 1:1 mixture of 1.5 M HClO4 and 2 M H3PO4 to 90 µl of extracts, followed by centrifugation. To determine total biopterins (BH4, BH2, and biopterins) by acid oxidation, 10 µl of 1% iodine in 2% KI solution was added to 90 µl protein-free supernatant. To determine BH2 and biopterins by alkaline oxidation, 10 µl of 1 M NaOH was added to 80 µl of extract, then 10 µl of 1% iodine/KI solution. Samples were incubated at room temperature for 1 hr in the dark. After incubation, Alkaline-oxidation samples were then acidified with 20 µl of 1M H3PO4. Excessive iodine was reduced by adding 5 µl of fresh ascorbic acid (20 mg/ml). Samples were centrifuged and 10 µl of supernatant was auto-injected in reverse phase HPLC system (Shimazu, Class-VP, Kyoto, Japan) equipped with a welchrom-C18 column (5 µm, 4.6×150 mm). The mobile phase was methanol–water (5:95, v/v) running at 0.8 ml/min. Fluorescence detection (350 nm excitation, 450 nm emission) was performed using an RF-10AXL (Shimazu, Kyoto, Japan). BH4 concentration, expressed as pmol/mg protein, was calculated by subtracting BH2+biopterin from total biopterins.

2.1.13 Statistical Analysis

Results are expressed as mean ± S.E.M. Statistical analyses were performed by one-way ANOVA with Dunnett test. Values of $p < 0.05$ were considered statistically different.
3 Chapter 3. Evaluation of physicochemical properties and drug potential of *Danshen* compounds

3.1 Introduction

The structure properties of a compound determine its physicochemical and biochemical properties, which drive the profiles of ADME/Tox in living system (Figure 1. 8). Unfavourable ADME/Tox profiles such as pharmacokinetic failure or toxicity are the major reasons for preventing drug candidate reaching the market or withdrawal of drug from the market at huge cost (803) (Table 1. 8). In order to avoid this situation, filtering out high risk compounds at the earliest stages of drug discovery is needed. To do this, it needs to take into account all physicochemical properties, such as logP, logS. These physicochemical properties are valuable in the prediction of ADME/Tox profiles of candidate compounds.

Drug-likeness aids in optimizing the ADME/Tox profile by analyzing the properties of a given compound. A number of molecular descriptors have emerged as reasonable informative and predictive, in particular, Rule-of-Five (Ro5), and been accepted and applied in drug discovery and development (792). The Lipinski’s Ro5 states that an orally active drug should respect: 1) a molecular weight < 500 Daltons, 2) a limited lipophilicity (expressed by LogP < 5, with P=[drug]org./[drug]aq.), 3) H-bond donors < 5 (expressed as the sum of OHs and NHs), 4) H-bond acceptors < 10 (expressed as the sum of Os and Ns). The Ro5 does not predict whether the compound is pharmacologically active, although it describes possible pharmacokinetics and properties of the compound in the human body, including ADME/Tox.

*Danshen* consists of over 100 of compounds showing a great diversity of physicochemical properties. Among of them, there are a number of compounds exerting pharmacological activities. However, so far, the relationship between physicochemical characteristics and drug potential has not been fully elucidated. Therefore, the work described in this chapter aims to analyse the physicochemical characteristics and properties for all known *Danshen* compounds using opened computational approaches.
3.2 Materials and methods

See Chapter 2, Section 2.1.2 to 2.1.5.
3.3 Results

3.3.1.1 Physicochemical property of Danshen compounds

The physicochemical properties of Danshen compounds are summarized in Table 3.1. There were 64 lipophilic compounds and 34 hydrophilic compounds evaluated. The distribution of clogP and clogS were graphed in Figure 3.1 and Figure 3.2. The value of logP was from -0.46 to 6.87; the range of logS was from -9.9 to 5.06. The molecular weight ranged from 138 to 746 Da.
Table 3.1. Physicochemical properties of 94 collected Danshen compounds.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Compound name</th>
<th>Drug score</th>
<th>Drug-likeness</th>
<th>LogS</th>
<th>LogP</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cryptotanshinone</td>
<td>0.36</td>
<td>-7.06</td>
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<td>3.38</td>
<td>296</td>
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<tr>
<td>2</td>
<td>Danshenxinkun A</td>
<td>0.42</td>
<td>-1.27</td>
<td>-4.77</td>
<td>3.16</td>
<td>296</td>
</tr>
<tr>
<td>3</td>
<td>Danshenxinkun B</td>
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<td>0.91</td>
<td>-5.28</td>
<td>4.11</td>
<td>280</td>
</tr>
<tr>
<td>4</td>
<td>1,2 Didehydromiltirone Δ1,2</td>
<td>0.24</td>
<td>-7.61</td>
<td>-4.98</td>
<td>4.46</td>
<td>282</td>
</tr>
<tr>
<td>5</td>
<td>Dehydromiltirone</td>
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<td>4.43</td>
<td>280</td>
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<td>278</td>
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<td>15,16-Dihydrotanshinone I</td>
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<td>278</td>
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<td>3.09</td>
<td>280</td>
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<td>-5.44</td>
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<tr>
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All data were generated by OSIRIS Property Explorer.

Abbreviation: Da, Dalton; MW, molecular weight.
Figure 3.1. Distribution of calculated lipophilicity of 94 Danshen compounds. LogP was calculated by using OSIRIS Property Explorer.

Figure 3.2. Distribution of calculated solubility of 94 Danshen compounds. LogS was calculated by using OSIRIS Property Explorer.
3.3.1.2 Violation of Ro5

After analysing physicochemical properties, all tested Danshen compounds were categorized into drug-like or nondrug-like according to Ro5. Among ninety four Danshen compounds, there were eighteen compounds which violated Ro5 (Table 3.2). There were two lipophilic compounds, ferruginol and neo-przewaquinone A, violated the Ro5 with a logP value of 5.18 and 6.87, respectively. However, there were sixteen hydrophilic compounds broke Ro5, which was mainly due to the violation of molecular weight, to a lesser extent, H-bond donor and acceptor. Thus, Danshen compounds were druggable. The result was summarised in Table 3.2.
Table 3. 2. Violation of Ro5 among 94 collected Danshen compounds.

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All data were generated by OSIRIS Property Explorer and ACD/ChemSketch.

Abbreviation: H-bond, hydrogen bond; MW, molecular weight; Ro5, Rule-of-Five.
3.3.1.3 Toxicity risk assessment

The toxicity risks for the drug-like Danshen compounds were assessed on mutagenicity, tumorigenicity, irritating effects and reproductive effects. For lipophilic compounds, 15 compounds were predicted to be irritating, 3 compounds to be mutagenic and 1 compound to have reproductive effects. No lipophilic compound predicted to be tumorigenic. For hydrophilic compounds, 5 with mutagenicity, 2 with tumorigenicity, 3 with irritating effects and 1 with reproductive effects (Table 3.3). The majority of evaluated compounds had low toxicity and only a small number of compounds possessed high toxicity risk. Therefore, most 94 Danshen compounds were predicted as low toxicity (Figure 3.3).
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<td>L</td>
</tr>
<tr>
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<td>Caffeic acid</td>
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<td>Danshensu</td>
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<tr>
<td>71</td>
<td>Isoferulic acid</td>
<td>L</td>
</tr>
<tr>
<td>72</td>
<td>Isosalvianolic acid C</td>
<td>L</td>
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<td>75</td>
<td>Methyl rosmarinate</td>
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<td>Prolithospermic acid</td>
<td>L</td>
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<td>Protocatechualdehyde</td>
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<td>Protocatechuic acid</td>
<td>H</td>
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<td>79</td>
<td>Rosmarinic acid</td>
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<td>Vanillic acid</td>
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</table>

All data were generated by OSIRIS Property Explorer.

Abbreviation: H, high risk;  L, low risk; M, medium risk.
Figure 3.3. Potential toxicity risks for 94 collected *Danshen* compounds.
3.3.1.4 Drug potential

The drug-likeness scores calculated according to Equation 2.1 ranged from -13.38 to 1.78 (Table 3.4). The most compounds were distributed between -5 to 2. Among those compounds, isoferulic acid was the top scored compound; tanshindiol A had a lowest drug-likeness value. In comparison to traded drugs, the majority of drug-like Danshen compounds possessed a favourable drug-likeness value. We also calculated the drug score for 77 Danshen compounds using Equation 2.2 and Equation 2.3. The overall drug-likeness score ranged from 0.15 to 0.89 (Table 3.4). Danshensu and isoferulic acid were the top two ranked compounds with the score of 0.86 and 0.89, respectively. The lowest one was salvinal. Tan I, Tan IIA and CT possessed an acceptable drug score, which was 0.26, 0.27 and 0.36, respectively.
### Table 3.4. Drug potential of drug-like *Danshen* compounds.

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<th>Compound number</th>
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<th>Drug score</th>
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<td>Danshenxinkun A</td>
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<td>0.42</td>
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<td>3</td>
<td>Danshenxinkun B</td>
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<td>0.41</td>
</tr>
<tr>
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<td>1,2 Didehydromiltirone $\Delta$ 1,2</td>
<td>-7.61</td>
<td>0.24</td>
</tr>
<tr>
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<tr>
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<td>Didydroisotanshinone I</td>
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<td>0.58</td>
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<tr>
<td>7</td>
<td>15,16-Dihydrotanshinone I</td>
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<td>0.37</td>
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<tr>
<td>8</td>
<td>15,16-Dihydrotanshinol B</td>
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<td>0.42</td>
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<tr>
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<td>Dihydrotanshinone I</td>
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</tr>
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<td>1,2-Dihydrotanshiquinone</td>
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<td>1,2-Dihydrotanshinone $\Delta$ 15,16</td>
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<td>0.33</td>
</tr>
<tr>
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<td>6,12-Dihydroxyabieta-5,8,11,13-tetraen-7-one</td>
<td>-5.15</td>
<td>0.31</td>
</tr>
<tr>
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<td>7a-hydroxyallyl-royleanone</td>
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<td>0.4</td>
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<td>3-Hydroxycryptotanshinone</td>
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All data were generated by OSIRIS Property Explorer.
3.4 Discussion

In this study, the physicochemical properties of 64 lipophilic and 30 hydrophilic Danshen compounds were analysed. There were 80 compounds categorized into drug-like compound. The majority of those drug-like compounds possessed a comparable profile of drug-likeness and drug score to the traded drugs. In addition, potential toxicity risk analysis revealed that the irritating effect was the main potential toxicity risk for the Danshen compounds.

There are many approaches to assess a compound's drug-likeness based on topological descriptors, fingerprints of MDL structure keys or other properties as cLogP and molecular weights. In this study, we used OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/) to evaluate drug-likeness. The approach is based on a list of about 5300 distinct substructure fragments with associated drug-likeness scores. The drug-likeness values of Danshen compounds ranged from -13.38 to 1.78. Compared to 3300 traded drugs (http://www.organic-chemistry.org/prog/peo/druglikeness.html), most compounds possessed a favourable distribution. A positive value implies that a compound contains predominantly fragments which are frequently present in commercial drugs. It doesn't necessarily mean that these fragments have been well ballanced against other properties of test compounds. For instance, a molecule may be composed of drug-like, but for lipophilic fragments only. It may have a high drug-likeness score but may be failed for being a drug because of its high lipophilicity. The overall drug-likeness scores represent drug potential of a given compound. It is a combination of logP, logS, drug-likeness and toxicity risk. According to Equation 2.2 and Equation 2.3, the drug score is ranging from 0 to 1. In our study, the tested Danshen compounds scored from 0.15 to 0.89.

It has been established that physicochemical property of a compound, such as logP and logS determines its ADME/Tox profile (779). LogP is a well-established measure of the compound's lipophilicity. High logP values or high lipophilicities indicates a poor absorption or permeation. It has been shown that for compounds to have a reasonable probability of being well absorbed their logP value must not be greater than 5.0 (792). The logP value has been relevant to ADME profile, such as
oral bioavailability, blood brain barrier partitioning, intestinal absorption, and plasma protein binding. For the 94 Danshen compounds evaluated, only two lipophilic compounds had a logP value over 5.0. This suggests that the most Danshen compounds exhibit a favourable lipophicility. Likewise, the aqueous solubility of a compound significantly affects its absorption and distribution characteristics. Typically, drugs with a low solubility are likely to have a low absorption. LogS is relevant to oral bioavailability, blood brain barrier partitioning and plasma protein binding (804). More than 80% of the traded drugs have a logS value greater than -4.

In our study, 64 out of 94 compounds are lipophilic. Thus, the overall logS distribution was shifted to left and the logS of most compounds ranged from -6 to -4, indicating that most compounds may have a relative low absorption.

Molecular weight is a crucial structural parameter (779). It has been showed that an increase in molecular weight leads to a decrease in solubility, permeability and distribution, which in turn result in a unfavourable profile of ADME (779). On the other hand, optimizing compounds for high activity on a biological target often goes along with increased molecular weights (779). Thus, to balance the ADME profile and efficacy, it often tries to keep molecular weights as low as possible. It has been showed that more than 80 % of all traded drugs have a molecular weight below 450.

In the present study, among 64 lipophilic compounds, only neo-przewaquinone A had a molecular weight of 542. For hydrophilic compounds, molecular weight is the main reason for the violation of Ro5. Almost 50% evaluated hydrophilic compounds had a molecular weight over 500. Nevertheless, molecule weight is not the only factor determining the drug-like property of a compound; it should be considered with other properties such as bioactivity and ADME.

Toxicity risk is the most likely reason for a drug withdrawn from the market or preventing a drug candidate reaching to market (805). Therefore, it is necessary to test the toxic effects of drug candidates in the early stage of drug development. In order to ensure the fast and reliable result, computational approaches have been employed to evaluate the potential toxicity risk (806). In our study, the predicted toxicity results showed that most drug-like Danshen compounds had a low toxicity, but irritating effect may be the major potential toxicity risk. This toxicity assessment
is consistent with the fact that certain *Danshen* compounds, such as tanshinones, have been clinically used as drugs to treat various diseases or conditions (642, 807).

In a summary, the findings from this study indicate that the majority of *Danshen* compounds possess a favourable druggable potential. Understanding the physicochemical properties of *Danshen* compounds may help to understand the mechanisms of actions of *Danshen* and also provide a basis for further development of better effective drugs derived from *Danshen* compounds.

![Distribution of drug-likeness values of *Danshen* compounds.](image)

**Figure 3. 4.** Distribution of drug-likeness values of *Danshen* compounds.
4 Chapter 4. Evaluation of biological fate, toxicity and drug interaction of natural products

4.1 Introduction

In recent years, there has been a significant increase in the use of herbal medicines to manage various chronic diseases or to promote health in many Western countries including Australia, New Zealand, the U.S. and Europe (808). To date, more than 11,000 species of plants for medicinal use have been documented and about 300 of them are commonly used (809). An estimated one third of adults in developed countries and more than 80% of the population in many developing countries use herbal medicines for promoting health or managing various diseases ranging from common cold, inflammation, heart disease, liver cirrhosis, diabetes to central nervous system diseases (810). This has occurred despite a lack of clinical evidence for the efficacy, targets and safety data of most commonly used herbal medicines.

There is a significant gap between the high demand in usage of herbal medicines and low regulatory controls on these products. Quite often, the ADME/Tox of herbal remedies is not required by regulatory authorities. However, this does not mean this issue is not important. To optimize the use of herbal remedies, including dosage, regimen and administration route, it is needed to examine their biological fate including the disposition pathways and kinetics in the human body. Only based on this knowledge, we can establish the potential dose-effect and dose-concentration relationships, which would allow us to conduct proper therapeutic monitoring for herbal remedies. Pharmacokinetic studies of herbal remedies also provide important information of potential herb-herb and herb-drug interactions. On the other hand, the unknown safety profiles of some herbal remedies may cause organ injuries in humans without knowing the underlying mechanisms. Herbal consumption has sometimes been associated with toxicities of the heart, liver, blood, kidney, central nervous system, and skin and less frequently carcinogenesis (811-817). Indeed, herb induced organ toxicities are often associated with herbal bioactivation in the body, possibly resulting in toxic species from the herbal remedies administered to the patients (818). In this regard, understanding the biotransformation pathways of
herbal remedies is important to explore the biochemical mechanisms of herbal toxicities.

Herbal medicines are often co-administered with therapeutic drugs, raising the potential of pharmacokinetic and/or pharmacodynamic herb-drug interactions. Clinically, a number of herb-drug interactions have been documented and many of them resulted in significant alterations in efficacy and/or adverse events (819-824). Herb-drug interactions are due to altered ADME of drugs. Induction or inhibition of hepatic and intestinal CYPs is one of the underlying mechanisms of altered drug concentrations at sites of action by co-administrated herbal medicines. Particularly, the mechanism-based inhibition of CYPs by herbal compounds may have important pharmacokinetic implications, in particular CYP3A4 (825, 826).

Thus, it is very important to evaluate the biological fate, toxicity and drug interaction of natural products. Recently, there is an increasing interest in using in silico approaches to study the biological fate, toxicity and drug interaction of natural products. There has been a significant progress in this field, although the evidence accumulated has not been properly reviewed. To update our knowledge on this, this chapter describes a literature review to highlight the pharmacokinetic properties and disposition pathways of plant natural products, and also discuss the relevant clinical and toxicological implications.
4.2 Data search

To retrieve relevant data, the authors have searched through computer-based literatures by full text search in Medline (via Pubmed), ScienceDirect, Current Contents Connect (ISI), Cochrance Library, CINAHL (EBSCO), CrossRef Search and Embase (all from inception to 28/08/2012). Keyword search terms included structure based computational analysis, ligand based computational analysis, virtual screening, high throughput screening, cytochrome P450, drug metabolizing enzyme, drug transporter, nuclear receptor and herbal medicine together with combination terms including drug interaction, drug design and in silico approaches.
4.3 Methods used in in silico approaches

In recent years, the application of in silico approaches in modeling ADME/Tox properties has been receiving increased attention in order to minimise or reduce the risk in late stage attrition in drug discovery and development process (827-829). Various computational methods for predicting the profile of ADME/Tox, drug interactions, drug targets for new chemical entity, potential drug or natural compounds have been developed (788, 791, 830, 831). Currently, a wide range of computational methods are available, ranging from hit identifications to lead optimization and beyond (832-834) The most commonly used techniques include ligand- (833) or structure based virtual screening (835). There are a number of commercially available ligand and protein based in silico methods, such as DOCK, Autodock, FlexX and GOLD.

Ligand-based virtual screening relies on chemical structure and characteristics of the molecules to identify the interaction between the compound and the target (i.e. CYPs, UGTs & transporters) (836, 837). These approaches include quantum mechanical methods, various descriptor based methods (e.g., classificators, quantitative structure activity/property relationships (QSPR) or quantitative structure activity relationships (QSAR) and pharmacophore generation. Based on the assumption that the biological fate of a compound mainly depends on its physicochemical properties with the involvement of drug metabolizing enzyme, transporters and receptors, models developed by this method are largely based on the structural information of the molecules, without taking into account the information of the target.

On the other hand, structure-based methods use the structure information solved by experimental protein structures and/or homology models, and often adopt automated docking approaches to identify the binding site of protein (838) and assess the binding affinity (835, 839, 840). For example, it is now capable of determining the binding mode of particular human CYPs in complex with various compounds and their probable metabolite(s) for predicting possible CYP mediated drug interactions. Similar the structure based methods have been used for studying other valid therapeutic target proteins such as P-gp, NOS and aromatase.
In addition, other computational approaches have also been used to predict the biological fate and toxic profile of a compound such as rule based methods and the ligand-protein interaction based approaches. Rule based approaches use data mining techniques based upon large databases to predict drug metabolism. The mixed approach is the combination of information related to both structure of ligands and proteins for better predictions on the biological fate and toxic profile of a compound.
4.4 Biological fate of natural compounds

Biological fate of natural compounds has been recognized as one of the most challenging areas investigating the complex biological process with the involvement of complex molecules, enzymatic systems, transporters, and receptors. Over 95% drugs and some natural products undergo Phase I and/or Phase II metabolism following excretion through renal route, to a lesser extent, fecal route, and respiratory route. Phase I reactions including oxidation, reduction and hydrolysis are mainly catalyzed by CYPs, which introduce new groups to a substance and make it more susceptible to Phase II conjugative reactions, which are carried out by flavin-containing monooxygenases, epoxide hydrolase, carboxylesterase and amidase, peroxidase, alcohol/aldehyde dehydrogenase, monoamine oxidase, or NADPH quinone reductase. Phase II reactions, such as glucuronidation, which is catalyzed by various UGTs and sulfation, which is catalyzed by sulfotransferases (SULTs), generally yield molecules, which are more amenable to biliary or renal excretion.

Drug transporters such as P-gp plays a critical role in absorption and excretion of a compound, thus have a major influence in oral bioavailability and half-life of natural compounds. Nuclear receptors, such as CAR, have an important role in drug disposition and are capable of regulating drug metabolizing enzymes such as CYPs. Thus, identification of actions of drug candidates on drug metabolizing enzymes, drug transporters and nuclear receptors will have important implication in predication of the ADME profile of drugs and natural products.

In silico ADME studies have been developed in a wide range of models such as modeling CYPs (789-791), UGTs (841), P-gp (842, 843), OCTs (844, 845), CAR (846) and PXR (788).

4.4.1 Phase I enzymes

CYP is the major Phase I drug metabolizing enzyme family accounting for metabolism of more than 95% drugs and some natural products. It is one of the major determinants of the metabolic kinetics of a substance, and plays a critical role in drug interactions and metabolism-dependent toxicity. The human CYP superfamily contains 57 functional genes and 58 pseudogenes (http://drnelson.utmem.edu/CytochromeP450.html, access date: 28/08/2012). CYP1,
CYP2 and CYP3 are responsible for the metabolism of over 90% of clinical drugs, while other subfamilies (e.g. CYPs 4, 7, 11, 17, 19, and 21) also involved in the metabolism of some endogenous including steroids, bile acids, and eicosanoids (847, 848). In particular, CYP1A1, 2D6, 2C9, 3A4 are the most important CYP isoforms in metabolism of drugs and natural compounds. Table 4.1 summarized all in vitro studies on metabolism of natural compounds involving CYPs. A large number of natural compounds, such as curcumin and Tan IIA can be metabolized by different CYP isoforms. CYP related mechanism is important for the pharmacological action of natural compounds, however, in some cases, it is also related to some toxic effects of natural compounds (849).
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Sources</th>
<th>CYP isoforms</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aristolochic acid*</td>
<td>Aristolochia fanchi</td>
<td>CYP1A1 &amp; 1A2</td>
<td>(850,</td>
</tr>
<tr>
<td>Bergamottin*</td>
<td>Grapefruit juice</td>
<td>CYP3A4, 2B6 &amp; 3A5</td>
<td>851)</td>
</tr>
<tr>
<td>Capsaicin*</td>
<td>Hot peppers</td>
<td>CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 &amp; 3A4</td>
<td>(853)</td>
</tr>
<tr>
<td>Coumarin*</td>
<td>Cinnamon, cassia, lavender &amp; peppermint</td>
<td>CYP1A1, 1A2, 2B6, 2E1 &amp; 3A4</td>
<td>(854,</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Curcuma longa</td>
<td>CYP2D6</td>
<td>855)</td>
</tr>
<tr>
<td>Diallyl sulfide</td>
<td>Garlic</td>
<td>CYP2E1</td>
<td>857)</td>
</tr>
<tr>
<td>5,4’-Dimethoxyisoflavone</td>
<td>Plant flavonoid</td>
<td>CYP1A2</td>
<td>858)</td>
</tr>
<tr>
<td>Emetine</td>
<td>Ipecac</td>
<td>CYP2D6 &amp; 3A4</td>
<td>859)</td>
</tr>
<tr>
<td>Emodin</td>
<td>Rhubarb</td>
<td>CYP1A2</td>
<td>860)</td>
</tr>
<tr>
<td>Estragole*</td>
<td>Nutmeg, tarragon, basil, anise, lemongrass, fennel, and pimento</td>
<td>CYP1A2, 2A6, 2C19, 2D6 &amp; 2E1</td>
<td>(861-863)</td>
</tr>
<tr>
<td>Formononetin</td>
<td>Plant flavonoid</td>
<td>CYP1A2</td>
<td>858)</td>
</tr>
<tr>
<td>Galangin</td>
<td>Cystus incanus L.</td>
<td>CYP1A1, 1A2 &amp; 2C9</td>
<td>864)</td>
</tr>
<tr>
<td>Genistein</td>
<td>Plant flavonoid</td>
<td>CYP1A1, 1A2, 1B1, 2E1 &amp; 3A4</td>
<td>(858,</td>
</tr>
<tr>
<td>Harmaline</td>
<td>Plant carboline alkaloid</td>
<td>CYP1A1, 1A2 &amp; 2D6</td>
<td>865)</td>
</tr>
<tr>
<td>Harmine</td>
<td>Plant carboline alkaloid</td>
<td>CYP1A1, 1A2, 2C9, 2C19 &amp; 2D6</td>
<td>866)</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Plant flavonoid</td>
<td>CYP1A2 &amp; 1B1</td>
<td>867)</td>
</tr>
<tr>
<td>Ibogaine</td>
<td>Tabernanthae iboga</td>
<td>CYP2D6</td>
<td>868)</td>
</tr>
<tr>
<td>Kempferide</td>
<td>Cystus incanus L.</td>
<td>CYP1A1, 1A2 &amp; 2C9</td>
<td>(864)</td>
</tr>
<tr>
<td>Lasiocarpine</td>
<td>Plant alkaloid</td>
<td>CYP2D6</td>
<td>869,</td>
</tr>
<tr>
<td>Limonene</td>
<td>Lemon</td>
<td>CYP2C9 &amp; 2C19</td>
<td>870)</td>
</tr>
<tr>
<td>Liquiritigenin</td>
<td>Plant flavonoid</td>
<td>CYP3A4 &amp; 3A5</td>
<td>872)</td>
</tr>
<tr>
<td>8-Methoxypsoralen*</td>
<td>Parsnips &amp; parsley</td>
<td>CYP2A6 &amp; 2B1</td>
<td>(873,</td>
</tr>
<tr>
<td>Menthofuran*</td>
<td>Pennyroyal oil</td>
<td>CYP2E1, 1A2 &amp; 2C19</td>
<td>874)</td>
</tr>
<tr>
<td>Methyleugenol*</td>
<td>Nutmeg, tarragon, basil, anise, lemongrass, fennel, and pimento</td>
<td>CYP1A2, 2C9, 2D6, &amp; 2C19</td>
<td>(876,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>877)</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>Plant alkaloid</td>
<td>CYP2D6</td>
<td>(869, 870)</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------</td>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>Paeonol</td>
<td>Moutan Cortex</td>
<td>CYP1A2</td>
<td>(878)</td>
</tr>
<tr>
<td>Prunetin</td>
<td>Plant flavonoid</td>
<td>CYP1A2</td>
<td>(858)</td>
</tr>
<tr>
<td>Pulegone*</td>
<td>Pennyroyal oil</td>
<td>CYP2E1, 1A2 &amp; 2C19</td>
<td>(875)</td>
</tr>
<tr>
<td><em>trans</em>-Resveratrol*</td>
<td>Grape seeds</td>
<td>CYP1A1, 1A2, &amp; 1B1</td>
<td>(879)</td>
</tr>
<tr>
<td>Rutaecarpine</td>
<td>Herbal alkaloid</td>
<td>CYP1A2, 2D6 &amp; 3A4</td>
<td>(880)</td>
</tr>
<tr>
<td>Safrole*</td>
<td>Nutmeg, mace, cinnamon, anise, black pepper, and sweet basil</td>
<td>CYP2A6, 2C9, 2D6 &amp; 2E1</td>
<td>(862, 881)</td>
</tr>
<tr>
<td>Silybin*</td>
<td>Milk thistle</td>
<td>CYP2C8</td>
<td>(882)</td>
</tr>
<tr>
<td>Tanshinone IIA</td>
<td><em>Danshen</em></td>
<td>CYP2A6</td>
<td>(883)</td>
</tr>
<tr>
<td>Teuchamaedryn A*</td>
<td>Germander</td>
<td>CYP3A4</td>
<td>(884-888)</td>
</tr>
<tr>
<td>Teucrin A*</td>
<td>Germander</td>
<td>CYP3A4</td>
<td>(884-888)</td>
</tr>
<tr>
<td>α-Thujone</td>
<td>Absinthe</td>
<td>CYP3A4</td>
<td>(889)</td>
</tr>
<tr>
<td>β-Thujone</td>
<td>Absinthe</td>
<td>CYP3A4</td>
<td>(889)</td>
</tr>
<tr>
<td>(-)-Verbenone</td>
<td>Rosemary species</td>
<td>CYP2A6 &amp; 2B6</td>
<td>(890)</td>
</tr>
</tbody>
</table>

*The asterisk indicates a potential for bioactivation to reactive intermediate by human CYPs.

Abbreviation: CYP: cytochrome P450.
With the elucidation of crystal structures of human CYPs, there is considerable interest in using in silico approaches to study CYP mediated metabolic profiles of new chemical entities and their interactions with CYPs, including toxicity and drug interactions (Table 4. 4). For example, a homology modeling has been successfully applied to study mammalian CYP1A1, 2A6, 2B6, 2C9 and 3A4 (891). Kemp et al. (892) used a homology modeling together with molecular docking approaches for studying human CYP2D6 and a range of substrates of CYP2D6. The method was able to discriminate between weak and tight binding compounds; and it was able to identify several novel inhibitors and the site of metabolism of the atypical substrate spirosulfonamide (892). Such computational studies have helped differentiating various substrates and inhibitors, and assisted in understanding their binding to the major human CYPs including CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A.

Recently, a number of new studies have provided insights into the molecular mechanisms of interaction of synthetic and herbal compounds of CYPs and identification of potential herb-drug interaction (788-791). Yang et al. (791) identified 19 out of the 56 natural compounds as potential inhibitors of CYP1A2. Mo et al. (790) identified 18 out of 40 compounds from S. baicalensis as potential inhibitors of CYP2D6.

4.4.2 Phase II enzymes
Phase II enzymes are responsible for the conjugation reactions, such as glucuronidation and sulfation and changes in these reactions may lead to altered drug efficacy and undesired effects. UGT is capable of metabolizing a number of drugs and natural compounds (Table 4. 2). Numerous natural compounds from different sources can be metabolized by UGT isoforms, such as UGT1A1 and 1A3. This conjugation reaction is related to the excretion of natural compounds, and in some cases, the detoxification of toxic natural compounds or their metabolites by increasing hydrophilicity. It is noted that FDA has highly encouraged evaluations of phase II enzymes if applicable (http://www.fda.gov).
Table 4.2. Natural compounds that are metabolized by human UGT enzymes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Sources</th>
<th>UGT isoforms</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alizarin</td>
<td>Plant anthraquinone</td>
<td>UGT1A3</td>
<td>(893)</td>
</tr>
<tr>
<td>Anthraflavic acid</td>
<td>Plant anthraquinone</td>
<td>UGT1A3</td>
<td>(893)</td>
</tr>
<tr>
<td>Apigenin</td>
<td>Plant flavonoid</td>
<td>UGT1A3, 1A8 &amp; 1A10</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>Aspalathin</td>
<td>Plant flavonoid</td>
<td>UGTs</td>
<td>(895, 896)</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>Plant phenolic compound</td>
<td>UGT1A8 &amp; 1A10</td>
<td>(894)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Plant polyphenolic compound</td>
<td>UGT1A8, 1A9, 1A10 &amp; 2B7</td>
<td>(897)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>Plant flavonoid</td>
<td>UGT1A1, 1A4, 1A6, 1A7 &amp; 1A9</td>
<td>(898, 899)</td>
</tr>
<tr>
<td>Emodin</td>
<td>Plant anthraquinone</td>
<td>UGT1A3, 1A8 &amp; 1A10</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>(-)-Epigallocatechin gallate</td>
<td>Tea catechin</td>
<td>UGT1A1, 1A8 &amp; 1A9</td>
<td>(900)</td>
</tr>
<tr>
<td>Esculetin</td>
<td>Plant coumarin derivative</td>
<td>UGT1A3 &amp; 1A8</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Plant phenolic compound</td>
<td>UGT1A3, 1A8 &amp; 1A10</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>Eupatilin</td>
<td>Artemisia plants</td>
<td>UGT1A1, 1A3, 1A7, 1A8, 1A9 &amp; 1A10</td>
<td>(901)</td>
</tr>
<tr>
<td>Farnesol</td>
<td>Plant isoprenoid</td>
<td>UGT1A1 &amp; 2B7</td>
<td>(902)</td>
</tr>
<tr>
<td>Fisetin</td>
<td>Plant flavonoid</td>
<td>UGT1A3, 1A8 &amp; 1A10</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>Galangin</td>
<td>Plant flavonoid</td>
<td>UGT1A1, 1A3, 1A9 &amp; 2B15</td>
<td>(893, 903)</td>
</tr>
<tr>
<td>Genistein</td>
<td>Plant flavonoid</td>
<td>UGT1A1, 1A4, 1A6, 1A7, 1A8, 1A9 &amp; 1A10</td>
<td>(893, 898, 899)</td>
</tr>
<tr>
<td>6-Gingerol</td>
<td>Ginger</td>
<td>UGT1A1, 1A3, 1A9 &amp; 2B7</td>
<td>(897, 904)</td>
</tr>
<tr>
<td>Luteolin</td>
<td>Plant</td>
<td>UGT1A1, 1A4, 1A8, 1A9, 1A10, 2B7 &amp; 2B15</td>
<td>(905)</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Plant flavonoid</td>
<td>UGT1A1, 1A3, 1A8, &amp; 1A10</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>Plant phenolic compound</td>
<td>UGT1A3</td>
<td>(893)</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>Plant flavonoid</td>
<td>UGTs</td>
<td>(906)</td>
</tr>
<tr>
<td>Protocatechuic aldehyde</td>
<td>S. miltiorrhiza</td>
<td>UGT1A6 &amp; 1A9</td>
<td>(907)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Plant flavonoid</td>
<td>UGT1A1, 1A3, 1A4, 1A8, 1A9, 1A10, 2B7 &amp; 2B15</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>Quinalizarin</td>
<td>Plant anthraquinone</td>
<td>UGT1A3</td>
<td>(893)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Grape seeds</td>
<td>UGT1A1, 1A9 &amp; 1A10</td>
<td>(908-911)</td>
</tr>
<tr>
<td>Scopoletin</td>
<td>Plant coumarin derivative</td>
<td>UGT1A3, 1A8, &amp; 1A10</td>
<td>(893, 894)</td>
</tr>
<tr>
<td>Scutellarin</td>
<td>S. radix (Huang qin)</td>
<td>UGTs</td>
<td>(912)</td>
</tr>
<tr>
<td>Thymol</td>
<td>Plant phenolic compound</td>
<td>UGT1A3</td>
<td>(893)</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>Plant coumarin derivative</td>
<td>UGT1A3</td>
<td>(893)</td>
</tr>
</tbody>
</table>

Abbreviation: UGT: uridine diphosphate glucuronosyltransferase.
There is an increasing number of *in silico* studies reported on structural characteristics of the ligands, which are able to influence molecular recognition and metabolism by the Phase II metabolizing enzymes (*Table 4. 4*).

For example, several studies have used computational methods to study the features of the ligands for UGT, including pharmacophore elucidation, two/three dimension quantitative structure activity relationships (2/3D-QSAR), and non-linear pattern recognition techniques. Said *et al.* (913) firstly reported a 3D-QSAR study of the rat liver bilirubin UGT using comparative molecular field analysis (CoMFA). In this study, 18 compounds with related structures to phenolphthalein and a triphenylalkylcarboxylic acid were examined as inhibitors (913). The 2/3D-QSAR and pharmacophore approaches were also employed to model UGT1A1, UGT 1A4 and UGT1A9, which demonstrated the characteristics of ligands for the recognition and metabolism (914, 915). These findings provide useful information on discriminating UGT substrates and inhibitors, identifying the characteristics of ligands and their molecular recognition and/or metabolism by UGT, which can be used to qualitatively and quantitatively predict the drug glucuronidation parameters.

### 4.4.3 Drug transporters

Drug transporters, including P-gp, OCTs, bile acid transporters, nucleoside transporters, human proton-coupled small peptide carrier (hPEPT1) and organic anion-transporting polypeptide (OATP), play an important role in the process of ADME and drug toxicity. It has been shown that many natural compounds, such as berberine, Tan I, Tan IIA and CT, are substrates of P-gp (*Table 4. 3*). This mechanism is related to the absorption, distribution and excretion of natural compounds, which in turn affects their pharmacological actions.
Table 4.3. Natural compounds that are reported to be substrates of P-gp/MDR1.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Sources</th>
<th>Test system(s)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baohuoside I</td>
<td><em>Epimedium koreanum</em> Nakai (Yin Yanghuo)</td>
<td>Caco-2 monolayers</td>
<td>(916)</td>
</tr>
<tr>
<td>Berberine</td>
<td>Berberis (e.g. <em>Hydrastis canadensis</em>)</td>
<td>Caco-2 monolayers</td>
<td>(917, 918)</td>
</tr>
<tr>
<td>Chrysophanol</td>
<td>Plant anthraquinone</td>
<td>Caco-2 monolayers</td>
<td>(919)</td>
</tr>
<tr>
<td>Cryptotanshinone</td>
<td><em>S. miltiorrhiza</em></td>
<td></td>
<td>(920)</td>
</tr>
<tr>
<td>Emodin</td>
<td>Rhubarb</td>
<td>Caco-2 monolayers</td>
<td>(919)</td>
</tr>
<tr>
<td>Ginsenoside Rh2</td>
<td>Ginseng</td>
<td>Caco-2 monolayers</td>
<td>(921)</td>
</tr>
<tr>
<td>Glabridin</td>
<td><em>Glycyrrhiza glabra</em> (licorice)</td>
<td>Caco-2 monolayers, MDR1 overexpressed cell line</td>
<td>(922)</td>
</tr>
<tr>
<td>Gomisin N</td>
<td><em>S. chinensis</em></td>
<td>Caco-2 monolayers</td>
<td>(923)</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>Plant flavonoid</td>
<td>Caco-2 monolayers</td>
<td>(924)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Plant flavonoid</td>
<td>Caco-2 monolayers</td>
<td>(924)</td>
</tr>
<tr>
<td>Matrine</td>
<td>Herbal alkaloid</td>
<td>Caco-2 monolayers</td>
<td>(925)</td>
</tr>
<tr>
<td>Protopanaxadiol</td>
<td>Ginseng</td>
<td>Caco-2 monolayers</td>
<td>(921)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Plant flavonoid (e.g. Ginkgo &amp; St John’s wort)</td>
<td>Caco-2 monolayers</td>
<td>(924)</td>
</tr>
<tr>
<td>Rutin deca (H-) sulfonate sodium</td>
<td>Flavonoid glycoside found in buckwheat</td>
<td>Caco-2 monolayers</td>
<td>(926)</td>
</tr>
<tr>
<td>Tanshinone I</td>
<td><em>S. miltiorrhiza</em> (Danshen)</td>
<td>Caco-2 monolayers, MDR1 overexpressed cell line</td>
<td>(927)</td>
</tr>
<tr>
<td>Tanshinone IIA</td>
<td><em>S. miltiorrhiza</em> (Danshen)</td>
<td>Caco-2 monolayers, MDR1 overexpressed cell line</td>
<td>(927)</td>
</tr>
<tr>
<td>Tanshinone IIB</td>
<td><em>S. miltiorrhiza</em> (Danshen)</td>
<td>Caco-2 monolayers, MDR1 overexpressed cell line</td>
<td>(927)</td>
</tr>
</tbody>
</table>

Abbreviation: MDR1, multiple drug resistance protein 1.
To date, *in silico* methods have been used for modeling the transporter’s 3D structure to assist in studying the drug transport process, discriminating substrate or inhibitor, and measuring the activity (Table 4.4). Currently, *in silico* approaches include transporter-based methods, such as homology modeling and structure-based methods, such as pharmacophore and 3D-QSAR modeling. Shilling et al. (843) successfully established a 3D model of LmrA as a homology for human multidrug transporter P-gp and found that all three available ABC transporters shared the same overall transmembrane structure.

Recently, substrate-based methods such as 3D-QSAR and pharmacophore modeling also has been applied in studying P-gp (929-931), OCTs (932), bile acid transporters, nucleoside transporters (933), hPEPT1 (934, 935), OATPs (844, 845). Crivori et al. (836) developed two computational models for identifying P-gp substrates and inhibitors. One method was based on calculated molecular descriptors and multivariate analysis using a training set of 53 diverse drugs for discriminating P-gp substrates and nonsubstrates. The model was capable of predicting the feature of 72% of an external set of 272 proprietary compounds by correlating to the experimental classes and using partial least squares discriminant (PLSD) (836). Moreover, PLSD analysis using GRIND-pharmacophore-based descriptors was conducted to model P-gp substrates with poor or no inhibitory effect vs inhibitors with no evidence of significant transport. This model is capable of identifying some key molecular features discriminating between substrates and inhibitors with an average accuracy of 82% (836). These computational approaches may help for identifying the substrate and inhibitor of transporter and designing new drug with favorite bioavailability and minimum drug resistance.

### 4.4.4 Nuclear receptors

Nuclear receptors play an important role in regulating the expression of the drug metabolizing enzymes and drug transporters such as CYP, CAR and PXR (936). There is evidence showing that a number of natural compounds are able to regulate the activity and expression of nuclear receptors. Liu et al (788) showed that physcion, protocatechuic aldehyde, Sal B, and sodium danshensu significantly increased the expression PXR mRNA, whereas, epifriedelanol, morin, praeertorin D,
mulberroside A, Tan I, and Tan IIA significantly reduced the expression of PXR mRNA. This interaction between natural compounds and nuclear receptors may further alter downstream targets. For example, an increase in the activity or expression of PXR can result in a up-regulation of CYP3A4 expression.

Recent studies have used some advanced computational tools for screening compounds for nuclear receptors and providing valuable information for understanding the metabolism of xenobiotics and detoxification regulated by nuclear receptors (Table 4.4). Kublbeck et al. (846) identified 17 novel agonists of human CAR using a 3D pharmacophore and molecular docking approach with a sequential virtual screening procedure. These 17 agonists activated human CAR and then increased the expression level of CAR target CYPs and transporters. According to the crystallographic studies, the ligand binding domain of human PXR has a large and conformable binding pocket which can respond to compounds of diverse size and shape. Lemaire et al. (937) identified 9 agonists of human PXR using computational approach. Among the 9 agonists, compound 1-(2-chlorophenyl)-N-[1-(1-phenylethyl)-1H-benzimidazol-5-yl] methanesulfonamide (C2BA-4) showed a particularly activity on activation of PXR more potent than that of the reference compound 4-[2,2-bis(diethoxyphosphoryl)ethenyl]-2,6-ditert-butyl-phenol (SR12813) (937). The activation of PXR was also studied recently using computational approaches. Liu et al. (788) docked 19 herbal compounds into the ligand-binding cavity of PXR, observed that the binding was mainly through hydrogen bond formation and/or π-π interactions with the residues Ser247, Gln285, His407, and Arg401. These findings may help to screen new chemical entities and the rational drug design, or to predict potential drug interactions.
<table>
<thead>
<tr>
<th>Topic</th>
<th>Key aspects</th>
<th>Major purpose/findings</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolic alteration</strong></td>
<td>Metabolic stability; Identification of biotransformations and metabolites;</td>
<td>QSAR model were able to correctly predict the metabolic stability for 17 of 20 selected analogs with a prediction performance of 85%.</td>
<td>(938)</td>
</tr>
<tr>
<td></td>
<td>Metabolic reactions: ligand regioselectivities, rates and reactivities</td>
<td>About 80% of the test set and ~70% of an additional validation set were classified correctly as stable or unstable using proprietary data of up to 15000 compounds</td>
<td>(939, 940)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Used Gaussian processes and their intrinsic confidence estimator in predictive modeling for metabolic stability assessment in four different training set with 900-1900 compounds</td>
<td>(941)</td>
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<tr>
<td></td>
<td></td>
<td>Used MetaDrug to predict metabolites and the activity of the original compound and its metabolites.</td>
<td>(776)</td>
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<tr>
<td></td>
<td></td>
<td>K-PLS and possibly other similar machine learning methods (such as support vector machines) can be used to predict human drug metabolite formation in a classification manner with 61 and 79% prediction performance on different reactions</td>
<td>(942)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presented a new method which provided the cytochrome involved and the site of metabolism for any human CYP mediated reaction acting on new substrates.</td>
<td>(943)</td>
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<tr>
<td></td>
<td></td>
<td>Gave valuable information about important interactions of inhibitors and substrates with CYP2C9 using docking and the site of metabolism predictions.</td>
<td>(944)</td>
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<tr>
<td></td>
<td></td>
<td>Examined the MetaSite algorithm by comparing its predictions with experimentally characterized metabolites of statins produced by CYP with 77% of correct predictions.</td>
<td>(945)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compared MetaSite and the docking methods to predict the metabolic sites for CYP3A4-mediated metabolic reactions</td>
<td>(946)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Explored the enzyme-ligand interactions in CYP2D6 &amp; 3A4 homology models and crystal structures.</td>
<td>(947)</td>
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<tr>
<td></td>
<td></td>
<td>Developed a rapid semiquantitative model for predicting likely sites of CYP3A4-mediated metabolism on drug-like molecules</td>
<td>(948)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Predicted the metabolism by CYP2C9 using alignment and docking studies of a validated database of 70 substrates.</td>
<td>(949)</td>
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<tr>
<td></td>
<td></td>
<td>Predicted the rates and regioselectivity of CYPs-mediated reactions.</td>
<td>(950, 951)</td>
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<td>Predicted metabolite for para-substituted anisoles based on <em>ab initio</em> complete active space self-consistent field calculations.</td>
<td>(952)</td>
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<tr>
<td></td>
<td></td>
<td>Evaluated the reaction energetics for CYP-mediated reactions.</td>
<td>(953, 954)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constructed and evaluated models of CYP2E1 to predict metabolism from docking, molecular dynamics, and density functional theoretical calculations.</td>
<td>(955)</td>
</tr>
<tr>
<td>Binding to specific drug metabolizing enzymes and transporters</td>
<td>Studies and Methods</td>
<td></td>
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<tr>
<td>---------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------</td>
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<td><strong>Table 4.4. Continued.</strong></td>
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<tr>
<td><strong>Studied the stereospecific CYP-catalyzed hydroxylation of the C(5)-H((5-exo)) bond in camphor using a combined quantum mechanical/molecular mechanical approach.</strong></td>
<td>(956)</td>
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<tr>
<td><strong>Predicted regioselectivity in CYP450/3A4 mediated metabolism using a combined model.</strong></td>
<td>(957)</td>
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<tr>
<td><strong>Described differential enantioselectivity in methoxychlor O-demethylation by CYP2C enzymes using in silico methods.</strong></td>
<td>(958)</td>
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<tr>
<td><strong>Studied comparative homology modeling of human CYP1A1 and confirmed residues involved in 7-ethoxyresorufin O-deethylation by site-directed mutagenesis and enzyme kinetic analysis.</strong></td>
<td>(959)</td>
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<tr>
<td><strong>Predicted the dominant CYP450 enzyme in human drug biotransformation with structure-based methods.</strong></td>
<td>(960)</td>
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<tr>
<td><strong>Studied CYP2D6 and 3A4 mediated metabolic N-dealkylation reaction rates using quantitative structure-metabolism relationship modeling method.</strong></td>
<td>(961)</td>
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<tr>
<td><strong>Studied QSAR-based regioselectivity models for human CYP 3A4, 2D6, and 2C9.</strong></td>
<td>(962)</td>
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<tr>
<td><strong>Studied the metabolism of CYP2C9 and CYP2C19 for gliclazide using homology modeling and docking methods</strong></td>
<td>(963)</td>
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<tr>
<td><strong>Developed the first a pharmacophore model for inhibition of human CYP2D6.</strong></td>
<td>(964)</td>
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<tr>
<td><strong>Analysed CYP2D6 inhibitors using 3D/4D-QSAR models.</strong></td>
<td>(965)</td>
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<tr>
<td><strong>Analysed CYP3A4 substrates using 3D-QSAR model.</strong></td>
<td>(966)</td>
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<tr>
<td><strong>Analysed CYP2B6 substrates using 3D-QSAR model.</strong></td>
<td>(967)</td>
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<tr>
<td><strong>Developed and validated an in silico CYP profiler based on pharmacophore models.</strong></td>
<td>(968)</td>
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<tr>
<td><strong>Predicted affinities for CYP2C9 with multiple computational methods.</strong></td>
<td>(969)</td>
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<tr>
<td><strong>Studied the isoform specificity of CYP 3A4, 2D6, and 2C9 substrates using ligand-based models.</strong></td>
<td>(970)</td>
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<tr>
<td><strong>Predicted catalytic site and virtual screened CYP2D6 substrates by consideration of water and rescoring in automated docking.</strong></td>
<td>(971)</td>
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<tr>
<td><strong>Evaluated the binding orientations of testosterone in the active site of homology models for CYP2C11 and CYP2C13</strong></td>
<td>(972)</td>
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<tr>
<td><strong>Studied CYP2D6 inhibitor aryloxypropanolamines using 2D and 3D descriptors by QSAR model.</strong></td>
<td>(973)</td>
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<tr>
<td><strong>Studied CYP1A2 inhibitor flavonoids using 2D and 3D descriptors by comparative QSAR models.</strong></td>
<td>(974)</td>
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<tr>
<td><strong>Analysed CYP2D6 substrate interactions using computational methods</strong></td>
<td>(975)</td>
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<td>Table 4.4. Continued.</td>
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<tr>
<td><strong>Characterized molecular features of CYP2B6 substrates by 2D and 3D QSAR.</strong> (976)</td>
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<tr>
<td><strong>Identified new potent and selective CYP2B6 inhibitors using 3D-QSAR analysis</strong> (977)</td>
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<tr>
<td><strong>Studied active site architecture and ligand binding using homology model of 1alpha,25-dihydroxyvitamin D3 24-hydroxylase CYP24A1.</strong> (978)</td>
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<tr>
<td><strong>Predicted drug binding to CYP2D6 and identified a new metabolite of metoclopramide using in silico methods.</strong> (979)</td>
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<tr>
<td><strong>Studied CYP3A4 inhibitors using QSAR model.</strong> (980)</td>
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<tr>
<td><strong>Studied active site architecture and ligand binding using a homology model of human retinoic acid metabolising enzyme CYP26A1.</strong> (981)</td>
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<tr>
<td><strong>Studied CYP2D6 inhibition in the aryloxypropanolamine series using a 3D-QSAR model.</strong> (982)</td>
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<tr>
<td><strong>Studied the inhibition of human CYP1A2 oxidation of 5,6-dimethyl-xanthenone-4-acetic acid by acridines via a molecular modeling method.</strong> (983)</td>
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<tr>
<td><strong>Characterized the CYP3A4 active site by homology modeling</strong> (984)</td>
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<tr>
<td><strong>Characterized of the CYP2C8 active site by homology modeling</strong> (985)</td>
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<tr>
<td><strong>Studied quantitative binding models for CYP2C9 based on benz bromarone analogues.</strong> (986)</td>
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<tr>
<td><strong>Studied homology modeling of CYP3A4 and analysed typical CYP3A4 substrate interactions.</strong> (987)</td>
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<tr>
<td><strong>Studied the key members of the resorufin series with CYP2C5 derived models of human CYP1A1, CYP1A2, CYP2B6 and CYP3A4 using molecular modeling methods.</strong> (988)</td>
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<tr>
<td><strong>Studied the interaction of human cytochromes P450 CYP 2 family enzymes with their substrates using QSAR methods.</strong> (989)</td>
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<tr>
<td><strong>Analysed the inhibition of recombinant CYP3A4 activity by structurally diverse compounds using a genetic algorithm-combined partial least squares method</strong> (990)</td>
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<tr>
<td><strong>Demonstrated a molecular basis for sulfotransferase substrate specificity by molecular modeling and quantitative structure-activity relationship analysis.</strong> (991)</td>
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<tr>
<td><strong>Predicted UGT1A1 substrates using pharmacophore and quantitative structure activity relationship modeling.</strong> (914)</td>
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<tr>
<td><strong>Identified potential P-glycoprotein substrates and inhibitors using computational models.</strong> (836)</td>
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<tr>
<td><strong>Studied Km values for sulfation of diverse phenolic substrates by human catecholamine sulfotransferase SULT1A3 with CoMFA modeling of enzyme kinetics.</strong> (992)</td>
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<tr>
<td><strong>Studied serotonin and its membrane transporter SERT using computational approaches and implied for drug design.</strong> (993)</td>
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</tbody>
</table>
Table 4.4. Continued.

<table>
<thead>
<tr>
<th>Induction</th>
<th>Interactions with drug transporters and nuclear receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elucidated the inhibition requirements of the human OCTN2 using pharmacophore to mine data.</td>
<td>(994)</td>
</tr>
<tr>
<td>Identified FDA-approved drugs that inhibit human ASBT and derived computational models for ASBT inhibition.</td>
<td>(995)</td>
</tr>
<tr>
<td>Used pharmacophore-based method to discover novel human PEPT1 inhibitors.</td>
<td>(934)</td>
</tr>
<tr>
<td>Applied 3D-QSAR method to predict P-gp inhibitors and substrates.</td>
<td>(930)</td>
</tr>
<tr>
<td>Studied the structural requirement of modulator of P-gp using 3D-QSAR method.</td>
<td>(929)</td>
</tr>
<tr>
<td>Analysed peptide substrates of the mammalian H+/peptide cotransporter PEPT1 using 3D-QSAR method.</td>
<td>(935)</td>
</tr>
<tr>
<td>Applied QSAR to analyse the behavior of Oatp1a5 substrates in the binding site.</td>
<td>(845)</td>
</tr>
<tr>
<td>Studied the influence of molecular structure on substrate binding to the human organic cation transporter, hOCT1 using quantitative structure activity approaches.</td>
<td>(932)</td>
</tr>
<tr>
<td>Studied the molecular requirements of the human nucleoside transporters hCNT1, hCNT2, and hENT1 using pharmacophore models.</td>
<td>(933)</td>
</tr>
</tbody>
</table>

**Induction**

**Interactions with drug transporters and nuclear receptors**

- Analysed antibiotics that activate PXR and induce CYP3A4 in liver and intestine using a comprehensive *in vitro* and *in silico* analysis. (996)
- Predicted human PXR activation using machine learning methods and docking. (997)
- Discovered a highly active ligand of human PXR with pharmacophore modeling and virtual screening. (937)
- Identified 17 substituted sulfonamides and thiazolidin-4-one derivatives as agonists of human CAR through a sequential virtual screening procedure using a 3D pharmacophore and molecular docking approach. (846)
- Applied 3D-QSAR method to predict P-gp inhibitors and substrates. (930)
- Studied the structural requirement of modulator of P-gp using 3D-QSAR method. (929)
- Analysed peptide substrates of the mammalian H+/peptide cotransporter PEPT1 using 3D-QSAR method. (935)
- Applied QSAR to analyse the behavior of Oatp1a5 substrates in the binding site. (845)
- Studied the influence of molecular structure on substrate binding to the human organic cation transporter, hOCT1 using quantitative structure activity approaches. (932)
- Studied the molecular requirements of the human nucleoside transporters hCNT1, hCNT2, and hENT1 using pharmacophore models. (933)
Table 4.4. Continued.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysed antibiotics that activate PXR and induce CYP3A4 in liver and intestine using a comprehensive <em>in vitro</em> and <em>in silico</em> analysis.</td>
<td></td>
<td>(996)</td>
</tr>
<tr>
<td>Predicted human PXR activation using machine learning methods and docking.</td>
<td></td>
<td>(997)</td>
</tr>
<tr>
<td>Discovered a highly active ligand of human PXR with pharmacophore modeling and virtual screening.</td>
<td></td>
<td>(937)</td>
</tr>
<tr>
<td>Identified 17 substituted sulfonamides and thiazolidin-4-one derivatives as agonists of human CAR through a sequential virtual screening procedure using a 3D pharmacophore and molecular docking approach.</td>
<td></td>
<td>(846)</td>
</tr>
</tbody>
</table>

Abbreviation: ASBT: apical sodium-dependent bile salt transporter; CAR: constitutive androstane receptor; CoMFA: comparative molecular field analysis; CYP: cytochrome P450; FDA: food and drug administration; hCNT: human concentrative nucleoside transporter; hENT 1: equilibrative nucleoside transporter 1; hOATP: human organic anion-transporting polypeptide; hOCT: human organic cation transporter; P-gp: P-glycoprotein; PEPT: proton-coupled small peptide carrier; PXR: pregnane X receptor; QSAR: quantitative structure activity relationship; SULT: sulfotransferase.
4.5 Toxicity of natural compounds

There are numerous cases of toxicity induced drug development failure and withdrawn from market such as rofecoxib (cardiac toxicity) and ximelagatran (hepatotoxicity). Toxicity is the cause of around 30% of the dropouts during late drug development stages (998, 999). Therefore, implementing toxicity screening or testing as early as possible in the drug development process is important not only to save time and money, but also to reduce the risk to harm patients or consumers. Usually, toxicological tests for new compounds using conventional experimental methods are costly and time-consuming, moreover, there is difficulty in accessing and interpreting toxicological data \textit{in vitro} and \textit{in vivo} (1000-1002). Therefore, there is therefore an increased demand for new approaches to rapidly and reliably predict the toxicity of compounds in early stage of drug development. Computational approaches for toxicity prediction have been applied into drug development process for evaluating potential toxic effect of compounds (999, 1003, 1004).

4.5.1 Computational analysis

Natural products usually contain multiple ingredients with complex nature, which can cause hurdles for toxicity assessment. Thus, there is a need to develop new approaches for testing toxicological of natural products instead of using traditional \textit{in vitro} and \textit{in vivo} experiments. Arvidson \textit{et al} (1005) screened the toxicity of six well-known natural compounds (estragole, pulegone, aristolochic acid I, lipoic acid, 1-octacosanol, and epicatchin) with known human exposure, chemical metabolism and mechanism of action using QSAR modeling. The findings showed a very good agreement between the \textit{in silico} results and the experimental toxicity data (1005). In addition, virtual screening of herb-target predictions are available and prevalent. Ehman \textit{et al}. (1006) screened a large number of compounds over 192 Chinese herbs and formulas and identify the multi-target anti-inflammation compounds using pharmacophore assisted docking. Valerio \textit{et al}. (1007) successfully predicted the rodent carcinogenic potential of 101 naturally occurring chemicals in the human diet using high throughput QSAR predictive modeling.
4.6 Drug interactions

Alterations on the activities of drug metabolizing enzymes, transporters or nuclear receptors, in particular, CYPs, UGT, P-gp, and PXR, are the major causes of known drug-drug and drug-herb interactions. The FDA draft drug interaction guidance has recommended the following CYPs for routine assessment to identify potential CYP-mediated drug interactions: CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A. Alteration of the catalytic activity of CYPs may cause undesired drug effects or toxicity. Theoretically, a compound with effects on drug metabolizing enzymes or drug transporters has potential to interact with the substrate for those enzymes or transporters. For example, activation of nuclear receptors can cause induction of CYP3A4 and P-gp expressions (1008), resulting in changes of circulating level of substrate for CYP3A4 and P-gp expressions.

The application of in silico approaches to predict drug-drug interaction has been widely used to assess the effect of a compound on CYPs (1009, 1010). Classification of CYPs inhibitor by using in silico models can predict potential drug-drug interactions (1011-1016). For example, Vasanthanathan et al. (1011) classified 67% of 7000 compounds as CYP1A2 inhibitors. Distinguishing regulators from ligands of transporters may minimize potential drug interactions induced by altered activity of transporters (1017-1020). Yasuda et al. (996) docked dicloxacillin and nafcillin into PXR and found that both were able to activate PXR resulting in CYP3A4 induction, and those modeling results were found to be line with data from in vitro experiments.

Herb-drug interactions can cause serious adverse reactions resulting from modification of the activity of drug metabolizing enzymes, transporters and nuclear receptors by co-administrated drugs, herb medicines or other food supplements. For example, St John’s wort, garlic, ginseng, ginkgo may interact with a range of conventional drugs, including some clinically important drugs with very narrow therapeutic index to cause, in some cases, life threatening and lethal consequence (818). It is important therefore to identify, timely and precisely, the potential herb-drug interactions. Recently, Yang et al. (791) used molecular docking approach studied CYP1A2 inhibitors from 56 herbal compounds coupled with in vitro assays. Moreover, Mo et al. (790) screened a number of compounds from S. baicalensis, by
using pharmacophore and quantitative structure-activity relationship (QSAR) models, and found that 18 compound were inhibitors of CYP2D6. In addition, Liu et al. (788) showed that 19 tested herbal compounds were able to interact with PXR, indicating potential herb-drug interactions.
4.7 Challenges

The value of using computational approaches in studying ADME/Tox profiles of compounds has been well recognised, such as the ability of screening huge number of compounds without costly synthesis them, which significantly accelerates drug discovery and development process. By using these computational methods, it is possible to discover valuable information at early stage of drug discovery process, which assists in strategic planning of drug discovery and development process.

Although there have been significant developments in this area, there are still some challenges. For example, the *in silico* ADME/Tox prediction cannot completely replace the classic *in vitro* and *in vivo* experiments. In some cases, there is still lack of the true physiological representations of the chemical entity or drug candidates in *in silico* models, which limit the ability to quantitatively predict the situation *in vivo*, such as drug metabolizing enzyme and transporter interplay at various tissues (e.g., CYP3A4 and P-pg in the intestine vs in the liver), and the relationship between *in vitro* and *in vivo* ADME/Tox profiles. There is also lack of details analysis of predictive strength of various models developed so far, in particular on correlations between different parameters and endpoints. In addition, in many cases, there is a lack of reliable experimental data to generate proper computational model for further analysis of relationship between the *in vitro* and *in vivo* data. Obviously, there is a need for further studies on establishing and validating further proper models with less complexity, high predictability, and better correlations with available *in vivo* and *in vitro* data. Such models will have important application in drug discovery and drug development.

In conclusion, the use of computational approaches has significantly advanced and improved the drug development process and performance. The accurate predication the biological fate and toxicity of chemicals and drugs, including natural products by an integration of *in silico* approaches with well-established *in vitro* and *in vivo* methods will make an important contribution in natural product and drug research in the future.
5 Chapter 5. Establishment of high glucose induced eNOS uncoupling model in EA.hy926 cells

5.1 Introduction

Endothelial dysfunction has been implicated in the pathogenesis of cardiovascular and metabolic diseases (see Chapter 1, Section 1.2). Hyperglycaemia is a characterization of type 1 and type 2 diabetes and plays a key role in diabetes associated vascular complications (71, 1021). It is speculated that endothelial dysfunction is a consequence of metabolic alterations related to diabetes, particularly hyperglycaemia.

The relationship between diabetes and cardiovascular diseases has been extensively investigated (71, 72, 1021). It has been established that hyperglycaemia related changes of eNOS mechanism contribute to the vascular damages (402, 413). Studies have demonstrated that after the exposure of high glucose, ECs showed reduced NO production and increased ROS generation (402, 1022, 1023), with enhanced NF-κB activation, inflammatory gene expression, extracellular and intracellular adhesion molecules expression, and leukocyte recruitment (1024-1032).

Oxidative stress is a major risk factor in hyperglycemia related vascular damages (Chapter 1, Section 1.1). In vasculature, the activation of NADPH oxidases and eNOS uncoupling are the two important contributors to endothelium dysfunction. It has been shown that the activity and expression of NADPH oxidases are increased under high glucose exposure (1033-1040), NOX4, a major constitutive NADPH oxidase isoform expressed in the endothelium, is increased after high glucose exposure leading to excessive ROS generation and oxidative stress (1036, 1039, 1040). In addition, there is substantial evidence for ROS related changes of eNOS function and NO production/degradation (1041). For example, it has been shown that excessive ROS reduced NO generation and BH4 bioavailability (1042).

Thus, in order to study the effects of Danshen compounds on eNOS uncoupling, it is important to establish a proper cell model of eNOS uncoupling. Based on observations that endothelial dysfunction and eNOS uncoupling have been
demonstrated in metabolic and cardiovascular diseases, the present study was set out to investigate the effect of high glucose on eNOS coupling mechanisms in a human endothelial cell line EA.hy926.
5.2 Materials and methods

See Chapter 2, Section 2.2.1 and 2.1.6 to 2.1.13.
5.3 Results

5.3.1 Effect of high glucose on superoxide production

In order to identify the sources of superoxide production under high glucose condition, we firstly tested the effects of DPI (a flavin-containing oxidase inhibitor), L-NAME (a NOS inhibitor), and Apo, (a NOXs inhibitor), on superoxide production in EA.hy926 cells. Incubation of cells with a high glucose concentration (35 mM) for 24 or 48 hr significantly increased the superoxide production compared to those incubated with a low glucose concentration (5.5 mM) (Figure 5. 1). The high glucose induced increase in superoxide production was significantly inhibited by Apo (100 µM), L-NAME (100 µM) and DPI (5 µM). These results demonstrate that superoxide is almost completely generated by all flavin-containing oxidases, in particular, by NOXs and NOS under high glucose condition.
Figure 5. 1. Intracellular superoxide production in cultured EA.hy926 cells. Effects of high glucose, L-NAME (100 µM), Apo (100 µM) and DPI (5 µM) superoxide production was examined in EA.hy926 cells. Cells and cell homogenates were treated as described in Materials & Methods. Superoxide production was evaluated by 5 µM/L lucigenin-enhanced chemiluminescence assay. *, p<0.05 and **, p<0.01 versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.2 Effect of high glucose on NO generation

To determine whether the activity of eNOS was changed under high glucose condition, we determined the NO production in cells treated with high glucose for 24 and 48 hr. A significant lower level of NO generation was observed compared to the control (5.5 mM glucose), which was partly inhibited by the NOS inhibitor L-NAME (100 µM) (Figure 5. 2). The results in Figure 5. 1 and Figure 5. 2 demonstrated that eNOS uncoupling occurs with an increase in superoxide production and a decrease in NO generation in endothelial cell line under the high glucose condition.
Figure 5.2. NO production in cultured EA.hy926 cells. Effect of high glucose on NO production was examined in EA.hy926 cells. Cells were treated as described in Methods. The intracellular level of NO was measured using the fluorescent probe DAF-2-DA. *, \( p<0.05 \) and **, \( p<0.01 \) versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.3 Effect of high glucose on NOX expression

Since NOX4 is the dominant and constitutive superoxide producing enzyme in ECs, it is important to investigate the expression of NOX4 in the cell model. Figure 5.3 showed the effects of high glucose on expressions of NOX4 in EA.hy926 cells. When the cells were cultured in 35 mM glucose media for 24 or 48 hr, the expressions of NOX4 were significantly increased (up to more than 2-fold) compared to the control at 5.5 mM glucose (Figure 5.3). The results indicate that up-regulated expression of NOX4 occurs under the high glucose condition contributing to the excessive accumulation of superoxide in ECs.
Figure 5.3. Effect of high glucose on the expression of NOX4 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. A: a representative experiment showing high glucose (35 mM) induced increases in NOX4 expressions. B: the result of densitometric analyses of graphing the expression of NOX4. Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively. *, p<0.05 and **, p<0.01 versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.4 Effect of high glucose on eNOS expression

To confirm the eNOS uncoupling under the high glucose condition, we examined the change of eNOS forms (dimer and monomer). High glucose exposure did not significantly change the expression of the total eNOS, compared with that in low glucose condition. However, it significantly reduced the ratio of the active (dimer) to the inactive forms (monomer) of eNOS (by 50% after 48 hr high glucose treatment) (Figure 5. 4). These results demonstrate that high glucose induce the changes from dimeric to monomeric eNOS.
Figure 5.4. Effect of high glucose on the expression of eNOS in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The ratio of eNOS dimer to monomer decreased after high glucose treatment for 24 hr. A: A representative experiment showing high glucose (35 mM) induced decreases in expressions of eNOS dimer, monomer and total eNOS. B: Results of densitometric analyses graphing the ratio of eNOS dimer to monomer. *, p<0.05 and **, p<0.01 versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.5 Effect of high glucose on intracellular BH4 and BH2 levels

Given that BH4 is a crucial cofactor for eNOS function and coupling, we assessed the changes of intracellular biopterin concentrations in ECs under low and high glucose conditions. By measuring the levels of BH4 to BH2 and its ratio, we found that cells treated with high glucose for 24 hr and 48 hr showed a significant lower level of ratio of BH4 to BH2. Compared to the control cells exposed to 5.5 mM glucose, the ratio of BH4 to BH2 was reduced by 40% after cells were treated with high glucose (35 mM) for 48 hr (Figure 5.5). The results indicate that high glucose induces eNOS uncoupling by decreasing the ratio of BH4 to BH2.
Figure 5. Effect of high glucose on the ratio of BH4 to BH2 in cultured EA.hy926 cells. All the samples were treated as described in Methods. The ratio of BH4 to BH2 was markedly decreased in high glucose (35 mM) condition. *, $p<0.05$ and **, $p<0.01$ versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.6 Effect of high glucose on expressions of GTPCH1 and DHFR
The expressions of GTPCH1, the rate-limiting enzyme in BH4 synthesis, and DHFR, the enzyme responsible for the conversion of BH2 to BH4 were also significantly suppressed under the high glucose condition (Figure 5. 6 and Figure 5. 7).
Figure 5. 6. Effect of high glucose on the expression of GTPCH1 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The expression of GTPCH1 was inhibited by high glucose (35 mM). A: a representative experiment showing high glucose (35 mM) induced decreases in expressions of GTPCH1. B: Results of densitometric analyses graphing the expressions of GTPCH1.*, \( p<0.05 \) and **, \( p<0.01 \) versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
Figure 5.7. Effect of high glucose on the expression of DHFR. Cells and cell lysates were treated as indicated in Methods. The expression of DHFR was inhibited by high glucose (35 mM). A: a representative experiment showing high glucose (35 mM) induced decreases in expressions of DHFR. B: Results of densitometric analyses graphing the expressions of DHFR. *, $p<0.05$ and **, $p<0.01$ versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.7 Effect of high glucose on expression of HSP90

As HSP90 is an important chaperone regulating eNOS function and superoxide production. We examined the expression of HSP90 under the high glucose condition. When the cells were cultured in 35 mM glucose media for 24 and 48 hr, the expression of HSP90 was significantly decreased compared to the control at 5.5 mM glucose (Figure 5.8). The results demonstrate that a decrease in the expression of HSP90 is involved in the impaired eNOS function under high glucose condition.
Figure 5.8. Effect of high glucose on the expression of HSP90 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. A: A representative experiment showing high glucose (35 mM) induced decreases in expressions of HSP90. B: Results of densitometric analyses graphing the expressions of HSP90. *, p<0.05 and **, p<0.01 versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.3.8 Effect of high glucose on expression of PI3K

Since it has been reported that NOX expression is regulated by PI3K (1043), we further examined the influence of PI3K inhibition on the expression of NOX4 in high glucose treated cells. Figure 5.9 shows that wortmannin, a PI3K inhibitor, significantly inhibited the high glucose induced NOX4 expression. Figure 5.10 showed that the expression of PI3K was significantly increased by high glucose. These findings indicate that PI3K pathway plays a role in oxidative stress induced eNOS uncoupling under high glucose condition in human ECs.
Figure 5. 9. Effect of PI3K inhibitor (wortammanin, WM) on the expression of NOX4 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. WM (100 nM) significantly suppressed the expressions of NOX4 which were enhanced by high glucose. **: results from a representative experiment that was performed three times with equivalent results. B: results of densitometric analyses graphing the expression of NOX4. ##, p<0.01 versus low glucose group; **, p<0.01 versus high glucose group; ^, p<0.01 versus 24 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
Figure 5.10. Effect of high glucose on the expression of PI3K subunit in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The expression of PI3K was enhanced by high glucose. A: results from a representative experiment that was performed three times with equivalent results. B: results of densitometric analyses graphing the expression of p110β. **, p<0.01 versus 48 hr high glucose group. Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
5.4 Discussion

The aim of this study was to set up an experimental hyperglycaemia cell model of eNOS uncoupling using EA.hy926 cells treated with glucose, we found that eNOS uncoupling occurred with a decrease in NO generation and an increase in eNOS derived superoxide production with a reduced ratio of BH4 to BH2 inhibition of expressions of eNOS, GTPCH1, DHFR, and HSP90, whereas the expression of NOX4 and PI3K was up-regulated.

The finding from this study is consistent with previous reports demonstrating eNOS uncoupling under high glucose condition in ECs, with enhanced superoxide generation and decreased NO production (1022, 1044, 1045). Superoxide production in ECs is mainly derived from NOXs (4), but can also be generated from uncoupled eNOS (1046). Our findings that superoxide production under a high glucose condition was strongly inhibited by Apo and partially inhibited by L-NAME indicate that both NOXs and uncoupled eNOS are likely sources of superoxide generation observed. This is further confirmed by our finding that the ratio of active form of eNOS (dimer) to its inactive form (monomer) was decreased under the high glucose condition without changing the total expression of eNOS, indicating that eNOS was uncoupled (426).

NOXs are a complex family with at least seven members: NOX1 to NOX5 and Duox1 and Duox2 (1047). NOX4 is one of the dominant subunits of NOX and a constitutively active one expressing in ECs (4, 1048). The role of NOX4 in producing $\text{O}_2^-$ or $\text{H}_2\text{O}_2$ is not conclusive. Knockdown of NOX4 attenuated high glucose induced $\text{H}_2\text{O}_2$ production in diabetic mice (1039). Production of $\text{H}_2\text{O}_2$ from endogenously expressed NOX4 has been observed in rat aortic smooth muscle cells as well (1049). Other studies have also showed that down-regulation of NOX4 decreased $\text{O}_2^-$ level (1050, 1051). On the other hand, recent studies have also shown that inhibition of the expression of NOX4 can alleviate oxidative stress in diabetic mice (1039). Sedeek et al. (514) also demonstrated the critical role of NOX4-based NOX in glucose induced oxidative stress in type 2 diabetes. On the other hand, the protective action of NOX4 in vasculature has also been reported. Schroeder et al. (1052) showed that NOX4 prevented vasculature from endothelial dysfunction by
increasing the expression of eNOS and NO generation. Ray et al. (217) demonstrated a vasodilating effect of NOX4. The high glucose induced increase in the expression of NOX4 has been confirmed in our study, indicating its involvement in high glucose induced eNOS uncoupling in EA.hy926 cells. In addition, we also observed that wortmannin significantly decreased the expression of NOX4. It has been reported that NOX is regulated by PI3K, PKC and NF-κB pathways are involved in (44, 1043, 1053). Thus, the results indicated that PI3K pathway may be involved in the regulation of NOX4 expression in high glucose induced EA.hy926 cells. Further studies are necessary to elucidate the exact role of PI3K signaling pathway in the regulation of NOX4 expression.

BH4 is an essential cofactor for the eNOS function and an inadequate supply of cellular BH4 has been linked to endothelial eNOS uncoupling (430, 801, 1022, 1042). In the present study, we observed a significant lower intracellular level of BH4 under the high glucose condition, indicating the bioavailability of BH4 is reduced. Importantly, the ratio of BH4 to BH2 was reduced significantly after cells were treated with high glucose, which is consistent with previous observations that the relative concentration of BH4 and BH2 is important in determining the redox regulation of eNOS (1054). This reduction on BH4/BH2 ratio is likely to be due to suppressed activities of enzymes involved in BH4 synthesis or conversion. This is supported by the present finding that high glucose inhibited the expressions of GTPCH1 and DHFR, indicated that high glucose impaired both de novo biosynthetic and salvage pathways of BH4, leading to intracellular BH4 deficiency and eNOS uncoupling. Taken together, these results indicate that high glucose induces eNOS uncoupling by reducing the ratio of BH4 to BH2. The link between BH4 and PI3K pathway is not clear. It is possible that the changes of PI3K and NOX induced by high glucose may be responsible for reduction of BH4 bioavailability, but further study is necessary to elucidate the mechanism involved.

HSP90 has a regulatory effect on eNOS function (411, 1055, 1056). It has been reported that inhibition of HSP90 caused a reduction of eNOS expression and phosphorylation in HUVECs (1057, 1058), leading to eNOS uncoupling with a decrease in enzymatic activity and NO release (412). The finding of high glucose
decreased the expression of HSP90 supports the involvement of this protein in eNOS uncoupling. Thus, it is possible that increase in HSP90 expression may salvage eNOS function.

In summary, EA.hy926 cells under a high glucose exposure showed an eNOS uncoupling, with reduced NO production and increased superoxide production. High glucose also inhibited the activity and expression of NOX4, decreased the ratio of BH4 to BH2 and suppressed the expressions of GTPCH1, DHFR, and HSP90. PI3K signal pathway may be responsible for the up-regulation of NOX4. The study provides an insight into the mechanisms of high glucose induced eNOS uncoupling. Importantly, the study has successfully established a cell model which can be useful for studying the effects of Danshen compounds on eNOS uncoupling and endothelial dysfunction.
Chapter 6. Effect of Tanshinones on high glucose induced eNOS Uncoupling in EA.hy926 cells

6.1 Introduction
As mentioned as in Chapter 1, endothelial dysfunction has been implicated in the pathogenesis of important cardiovascular and metabolic diseases such as atherosclerosis, diabetes and obesity (56, 77, 78). The key mechanism of endothelial dysfunction is the imbalance of endothelium derived NO production and ROS generation, resulting in a decline in the bioavailability of NO and excessive accumulation of ROS (1, 78). This finally leads to oxidative stress and cellular injuries. In the vasculature, the main sources of ROS are those generated from NOXs, a family consisting of seven enzymes (NOX1-5, DUOX1 and DUOX2) which are composed of a number of regulatory and catalytic subunits (4, 308, 1048). Recent evidence indicates that NOX1, NOX2 and NOX4 play important roles in the regulation of cardiovascular functions (1, 1048). For example, NOX4-based NOX has been shown to mediate glucose induced oxidative stress in diabetes (514, 1059); and the overexpression of NOX2 potentiated vascular oxidative stress (104).

eNOS is a critical enzyme for the maintenance of cardiovascular function by producing NO. It catalyzes the formation of NO from L-arginine and O$_2$ in a reaction requiring several cofactors including Ca$^{2+}$/CaM, FAD, FMN, NADPH, and BH$_4$. However, under certain pathological circumstances and oxidative stress conditions, eNOS can be uncoupled, leading to generation of superoxide instead of NO (429-431). BH$_4$ is a crucial cofactor for the activity of eNOS. An inadequate supply of cellular BH$_4$ has been linked to eNOS uncoupling and endothelial dysfunction, whereas supplementation with BH$_4$ has been shown to improve eNOS coupling and endothelial function (430). Endogenous BH$_4$ is formed by a \textit{de novo} synthesis pathway and a salvage pathway that restore BH$_4$ from its oxidized form BH$_2$ (430, 431, 456, 1060). Studies indicate that the relative concentration of BH$_4$ over BH$_2$ plays a determining role in the redox regulation of eNOS-modulated endothelial responses since BH$_2$ itself can cause eNOS dysfunction (1054). Elevated eNOS expression without further increase in BH$_4$ level can also result in eNOS uncoupling due to an imbalance of eNOS enzyme and cofactors (1042). Other
mechanisms involved in eNOS uncoupling include S-glutathionylation of eNOS (415) and disruption of associations of eNOS with certain regulators such as HSP90 (411, 1055, 1056).

Tan I, Tan II and CT are the most abundant pharmacologically active tanshinones isolated from *Danshen*. The beneficial effects of these tanshinones on endothelial function have been extensively studied which are described in Section 1.7. However, it is still unclear whether these three major tanshinones affects eNOS uncoupling and if so, what mechanisms are involved. We hypothesize that Tan I, Tan IIA and CT can regulate eNOS uncoupling in ECs through targeting NOXs and cofactors of eNOS. In this part, we aimed to investigate the effects of Tan I, Tan IIA and CT on eNOS uncoupling induced by high glucose in human ECs and to identify possible intracellular molecular targets involved.
6.2 Materials and methods

See Chapter 2, Section 2.2.1 and 2.1.6 to 2.1.13.
6.3 Results

6.3.1 Effect of tanshinones on cell viability

First, the toxic effect of Tan I, Tan IIA and CT on EA.hy926 cells was determined by MTT assays. The cytotoxicity of these three tanshinones is in a concentration- and time-dependent manner in the tested range of 0.1-30µmol/L up to 48hr (Figure 6.1). Under these conditions, 0.08% DMSO had no cytotoxic effects on cells (data were not shown). Cytotoxicity of three tanshinones increased in relation to rising concentrations and incubation times as shown in. Tan I, Tan IIA and CT did not exert a cytotoxic effect in the range of 0.1-10µmol/L within 24 hr, whereas Tan I and Tan IIA had a significant cytotoxic effect at the concentration of 10µmol/L after incubating 48hr with a decrease in cell viability of 40%. At the concentration of 30µmol/L, three tanshinones significantly decreased the cell viability after incubating 24 and 48hr, respectively. Moreover, after 48 hr treatment, CT exerted a cytotoxic effect at the concentrations of 1 µmol/L and 10 µmol/L, respectively.
Figure 6. Cytotoxic effects of Tan I, Tan IIA, and CT on cultured EA.hy926 cells. Cytotoxic effects of Tan I, Tan IIA, and CT on ECs. EA.hy926 cells were seeded into 96-well plate at a density of 5000 cells/well. MTT assay was performed as described in Methods. Data represent the mean ± S.E. derived from five independent experiments. *, p<0.05.
6.3.2 Effect of tanshinones on high glucose induced superoxide production

In order to evaluate the effect of Tan I, Tan IIA and CT on superoxide production under eNOS uncoupling condition, we examined the effect of Tan I, Tan IIA and CT on high glucose induced superoxide production in EA.hy926 cell line. Tan I, Tan IIA and CT, in a concentration range (1-10 µM) which did not affect the cell viability as determined by MTT assay, significantly and concentration-dependently inhibited high glucose induced superoxide production (Figure 6.2). Tan I and Tan IIA at 1 µM and 3 µM, respectively produced a similar inhibition as that caused by L-NAME at 100 µM; Among these three tanshinones, CT showed the most effective inhibitory effect on superoxide production (Figure 6.2). These results demonstrate that Tan I, Tan II and CT has an inhibitory action on NOXs and NOS driven superoxide production under high glucose condition.
Figure 6.2. Effect of Tan I, Tan IIA and CT on superoxide production in cultured EA.hy926 cells. Cells and cell homogenates were treated as described in Materials & Methods. Superoxide production was evaluated by 5 µM/L lucigenin-enhanced chemiluminescence assay. (##, p<0.01 versus low glucose group; *, p<0.05 and **, p<0.01 versus 48 hr high glucose group). Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
6.3.3 Effect of tanshinones on high glucose induced inhibition of NO generation

Further to investigate the underlying molecular mechanism of tanshinones’ vascular protective effects, we tested the regulatory effect of Tan I, Tan IIA and CT on the activity of eNOS under high glucose condition. Tan I, Tan IIA and CT significantly increased the NO generation under high glucose condition with all tested concentrations, and the effect was abolished by L-NAME (100 µM) (Figure 6.3). From these results, we conclude that Tan I, Tan IIA and CT improve the function of eNOS which was impaired by oxidative stress under high glucose condition.
Figure 6. 3. Effect of Tan I, Tan IIA, and CT on NO production in cultured EA.hy926 cells. Cells were treated as described in Methods. The intracellular level of NO was measured using the fluorescent probe DAF-2-DA. (##, p< 0.01 versus low glucose group; **, p<0.01 versus 48 hr high glucose group). Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.3.4 Effect of tanshinones on NOX expression

Since NOX4 is the dominant and constitutive superoxide producing enzyme in ECs, we evaluated the effect of Tan I, Tan IIA and CT on NOX4 expression under high glucose condition. As we observed that the expressions of NOX4 were significantly increased (up to more than 2-fold) compared to the control at 5.5 mM glucose (Figure 5.3), when the cells were incubated with high glucose initially for 24 hr then treated with Tan I, Tan IIA and CT for another 24 hr, respectively, the expressions of NOX4 were markedly decreased, to a level similar to that at the low glucose (Figure 6.4). Moreover, compared to 24 hr high glucose treatment, Tan I, Tan IIA and CT down-regulated the expression of NOX4 significantly (Figure 6.4). The results indicate that Tan I, Tan IIA and CT can regulate the expression of NOX4 to exert its inhibition of superoxide production under the high glucose condition.
Figure 6.4. Effects of high glucose and Tan I, Tan IIA and CT on the expression of NOX4 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. A: a representative experiment showing high glucose (35 mM) induced increases in NOX4 expressions and the effects of Tan I, Tan IIA and CT. B: the result of densitometric analyses of graphing the expression of NOX4. (#, $p < 0.05$ and ##, $p < 0.01$ versus low glucose group; ^, $p < 0.01$ and **, $p < 0.01$ versus 24 hr and 48 hr high glucose group, respectively.). Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.3.5 Effect of tanshinones on eNOS expression

To confirm the eNOS uncoupling under the high glucose condition, we examined the change of eNOS forms (dimer and monomer). When the cells were firstly treated with high glucose for 24 hr and then received a 24 hr treatment of Tan I, Tan IIA and CT for another 24 hr, respectively, the ratio of eNOS dimer to monomer was recovered without significantly changes in total eNOS (Figure 6. 5). Furthermore, compared to 24 hr high glucose treatment, there was a significant increase in the ratio of eNOS dimer to monomer at high concentration of Tan IIA at 10 μM; whereas, Tan I and CT exerted the most beneficial action with a concentration of 3 and 1 μM, respectively. These results demonstrate that Tan I, Tan IIA and CT reverse the changes from dimeric to monomeric eNOS induced by high glucose exposure.
Figure 6.5. Effects of Tan I, Tan IIA and CT on the ratio of eNOS dimer to monomer in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The ratio of eNOS dimer to monomer decreased after high glucose treatment for 24 hr. Tan I, Tan IIA and CT significantly increased the ratio of eNOS dimer to monomer which was decreased by high glucose. A: A representative experiment showing high glucose (35 mM) induced decreases in expressions of eNOS dimer, monomer and total eNOS, and the effects of Tan I, Tan IIA and CT. B: Results of densitometric analyses graphing the ratio of eNOS dimer to monomer. Data represent the mean ± S.E.M. derived from three independent experiments (#, p < 0.01 versus low glucose group; *, p < 0.05 and **, p < 0.01 versus 48 hr high glucose group; ^, p < 0.01 versus 24 hr high glucose group.). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.3.6 Effect of tanshinones on intracellular BH4 and BH2 levels

Given that BH4 is a crucial cofactor for eNOS function and coupling, we examined the effect of Tan I, Tan IIA and CT on the changes of intracellular biopterin concentrations in endothelial cell line under high glucose condition. By measuring the levels of BH4 to BH2 and its ratio, we have already found that cells treated with high glucose for 24 hr and 48 hr showed a significant lower level of ratio of BH4 to BH2. When the cells were cultured in high glucose media initially for 24 hr then treated with Tan I, Tan IIA and CT for another 24 hr, the ratio of BH4 to BH2 was significantly increased with a high concentration of Tan I, Tan IIA and CT (10 µM) (Figure 6. 6). Compared to the other two tanshinones, Tan I had the most effective action on the increase in the ratio of BH4 to BH2. The results indicate that Tan I, Tan IIA and CT can reduce eNOS uncoupling by increasing the ratio of BH4 to BH2.
Figure 6. Intracellular levels of BH4 and BH2 in cultured EA.hy926 cells. All the samples were treated as described in Methods. The ratio of BH4 to BH2 was markedly decreased in high glucose (35 mM) condition. Tan I, Tan IIA and CT reduced the high glucose induced BH4 depletion in a concentration-dependent manner. Data represent the mean ± S.E.M. derived from three independent experiments (###, *p* < 0.01 versus low glucose; ***, *p* < 0.01 versus high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.3.7 Effect of tanshinones on expressions of GTPCH1 and DHFR

To determine whether the effect of Tan I, Tan IIA and CT on regulation of intracellular biopterin concentrations involves the *de novo* synthesis and conversion pathways, we examined the effects of Tan IIA on the expression of GTPCH1, the rate-limiting enzyme in BH4 synthesis, and DHFR, the enzyme responsible for the conversion of BH2 to BH4. Based on the effect of Tan I, Tan IIA and CT on the ratio of BH4 to BH2, we tested the effect of 10 µM Tan I, Tan IIA and CT. When the cells were cultured in high glucose media initially for 24 hr then treated with Tan I, Tan IIA and CT for another 24 hr, the expression of GTPCH1 and DHFR was significantly increased (Figure 6. 7), which were suppressed under high glucose condition. These results demonstrate that Tan I, Tan IIA and CT increases the ratio of BH4 to BH2 through enhancing the expression of GTPCH1 and DHFR.
Figure 6. 7. Effects of high glucose and Tan I, Tan IIA and CT on the expressions of GTPCH1 and DHFR in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The expressions of GTPCH1 and DHFR were inhibited by
high glucose (35 mM). Tan I, Tan IIA and CT (10 µM) significantly enhanced the expressions of GTPCH1 and DHFR which were suppressed by high glucose. A: a representative experiment showing high glucose (35 mM) induced decreases in expressions of GTPCH1, and the effects of Tan I, Tan IIA and CT. B: a representative experiment showing high glucose (35 mM) induced decreases in expressions of DHFR, and the effects of Tan I, Tan IIA and CT. C: Results of densitometric analyses graphing the expressions of GTPCH1 and DHFR. Data represent the mean ± S.E.M. derived from three independent experiments (#, \( p<0.05 \) and ##, \( p<0.01 \) versus low glucose group; **, \( p<0.01 \) versus high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.3.8 Effect of tanshinones on expression of HSP90

As HSP90 is an important chaperone regulating eNOS function and superoxide production. We examined the effect of Tan I, Tan IIA and CT on expression of HSP90 under the high glucose condition. When the cells were incubated with high glucose initially for 24 hr then treated with Tam I, Tan IIA and CT for another 24 hr, the expressions of HSP90 were significantly increased in a concentration-dependent manner (Figure 6. 8). The decrease in expression of HSP90 was significantly reversed by 3 and 10 µM of Tan I, Tan IIA and CT, compared to those at the high glucose condition at 24 and 48 hr. The results demonstrate that Tan I, Tan IIA and CT can reverse the impairment of HSP90 expression elicited by high glucose exposure, resulting in improved function of eNOS.
**Figure 6.8.** Effects of high glucose and Tan I, Tan IIA and CT on the expression of HSP90 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in *Methods*. A: A representative experiment showing high glucose (35 mM) induced decreases in expressions of HSP90, and the effects of Tan IIA. B: Results of densitometric analyses graphing the expressions of HSP90. Data represent the mean ± S.E.M. derived from three independent experiments (#, *p* < 0.05 versus low glucose group; *, *p* < 0.05 and **, *p* < 0.01 versus high glucose group; ^, *p* < 0.01 versus 24 hr high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.3.9 Effect of tanshinones on expression of PI3K

Since it has been reported that NOX expression is regulated by PI3K (1043) and we found that inhibition of PI3K caused a reduction in the expression of NOX4 (Figure 5. 9). Thus, we examined the effect of Tan I, Tan IIA and CT on the expression of PI3K in high glucose treated cells. When the cells were incubated with high glucose initially for 24 hr then treated with Tan I, Tan IIA and CT for another 24 hr, the expressions of PI3K were significantly decreased (Figure 6. 9). These findings indicate that PI3K pathway plays a role in oxidative stress under high glucose condition and it is involved in the actions of Tan II against eNOS uncoupling in human ECs.
Figure 6.9. Effects of Tan I, Tan IIA and CT on expression of PI3K in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. Tan I, Tan IIA and CT significantly suppressed the expressions of PI3K which were enhanced by high glucose. A: results from a representative experiment that was performed three times with equivalent results. B: results of densitometric analyses graphing the expression of p110β. Data represent the mean ± S.E.M. derived from three independent experiments (##, p<0.01 versus low glucose group; **, p<0.01 versus high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
6.4 Discussion

The aim of this study was to examine if Tan I, Tan IIA and CT, three main active constituent isolated from *S. Miltiorrhiza*, regulate eNOS uncoupling in EA.hy926 cells through targeting NOXs and cofactors of eNOS. Using the high glucose induced eNOS uncoupling cell model as described in Chapter 5, the effects of three main tanshinones on eNOS uncoupling were examined. We have focused on the therapeutic ability of Tan I, Tan IIA and CT, in endothelial dysfunction caused by high glucose exposure. The main finding is that high glucose induced eNOS uncoupling in EA.hy926 cells can be restored by Tan I, Tan IIA and CT. To the best of our knowledge this is the first report of the effects of tanshinone compounds on eNOS uncoupling in human ECs.

It has been reported that eNOS is a potential vascular target of *Danshen*. Tan IIA has been recently reported to regulate eNOS function by phosphorylation of eNOS to increase NO generation (1061). It is also reported that CT is capable of increasing NO production by upregulating eNOS expression in HUVECs (667). These compounds have reported with superoxide scavenging and NO stimulator properties in vascular ECs (642, 667, 1061-1065), anti-adhesion (640, 661, 662), and vasodilating effect (1066). The findings in this study showed that Tan I, Tan IIA and CT may act by regulating the dimerization of eNOS rather than increase total eNOS enzymes. This new finding may help for further understanding of the cardiovascular protective actions of *Danshen*, in particular, the beneficial actions of Tan I, Tan IIA and CT (600, 642, 1067, 1068).

NOXs are the primary source of superoxide in vasculature with at least seven members: NOX1 to NOX5 and Duox1 and Duox2 (61, 1047, 1048). As discussed in Chapter 5, NOX4 contributed to the superoxide production in high glucose treated EA.hy926 cells. The finding that Tan I, Tan IIA and CT inhibited the expression of NOX4 in high glucose treated EA.hy926 cells indicates that NOX4 may play a role in mediating the antioxidant action of Tan I, Tan IIA and CT by inhibiting ROS production via inhibition of NOX4. The exact mechanism of these tanshinones induced NOX4 inhibition is unclear. The regulation on NOXs has been reported involving PI3K, PKC and NF-κB pathways (44, 1043, 1053, 1069-1072). We also
observed that wortmannin significantly decreased the expression of NOX4, and Tan I, Tan IIA and CT suppressed the expression of PI3K which was increased under high glucose condition. These results, suggest that tanshinones may decrease the expression of NOX4 and restores eNOS uncoupling via, at least partially, PI3K pathway. Further studies are necessary to elucidate the role of PI3K signaling pathway in tanshinones induced regulation of NOX4 expression.

Further to the beneficial actions of three tanshinones on eNOS uncoupling, we found that Tan I, Tan IIA and CT increased the BH4/BH2 ratio indicates that it may improve eNOS uncoupling by increasing the bioavailability of BH4. BH4, which is an essential cofactor for the eNOS function. Inadequate supply of cellular BH4 has been linked to endothelial eNOS uncoupling (430, 801, 1022, 1042). The increase in bioavailability of BH4 by tanshinones is likely to be mediated by enzymes involved in BH4 synthesis or conversion. This is supported by the finding that Tan I, Tan IIA and CT counteracted the inhibitory effects of high glucose treatment on expressions of GTPCH1 and DHFR. The findings suggest that high glucose impaired both de novo biosynthetic and salvage pathways of BH4, leading to intracellular BH4 deficiency and eNOS uncoupling; Tan I, Tan IIA and CT may regulate BH4 synthesis to maintain adequate intracellular BH4 content by increasing the expressions of GTPCH1 and DHFR, and/or prevent BH4 from oxidation by its anti-oxidative effects. The enhancing effect of Tan I, Tan IIA and CT on the ratio of BH4 to BH2 in ECs has not been reported previously. We have also observed that when the cells were incubated with high glucose initially for 24 hr then treated with wortmannin for another 24 hr, the ratio of BH4 to BH2 was increased. Taken together, these results indicate that Tan I, Tan IIA and CT restore eNOS uncoupling by increasing the ratio of BH4 to BH2 which may also involve PI3K pathway.

Additionally, we also investigated the involvement of HSP90 in actions of Tan I, Tan IIA and CT, as it has been known that HSP90 has a regulatory effect on eNOS function (411, 1055, 1056). It has been reported that inhibition of HSP90 caused a reduction of eNOS expression and phosphorylation in HUVECs (1057, 1058), leading to eNOS uncoupling with a decrease in enzymatic activity and NO release (412). Thus, increase in HSP90 expression may salvage eNOS function with an
increase in its enzymatic activity and NO release. The finding that Tan I, Tan IIA and CT restored the decrease in the expression of HSP90 under high glucose condition indicates that these tanshinones may regulate eNOS function by acting on HSP90. It was noted that the actions of Tan I, Tan IIA and CT on HSP90 expression occurred at a lower concentration that its action on BH4 levels (Figure 6.8). Thus, it is possible that tanshinones may act on multiple mechanisms on restoring eNOS uncoupling, at low concentrations acting through regulating of interactions of HSP90 with eNOS. At a higher concentration, it may regulate expressions of GTPCH1 and DHFR and subsequent BH4 levels. Further study is necessary to elucidate the mechanism involved in Tan I, Tan IIA an CT’s effect on regulation of HSP90-eNOS interaction. It should be pointed out that the present study does not exclude the possibility of involving other mechanisms such as S-glutathionylation of eNOS (415) in protection of Tan I, Tan IIA and CT against eNOS uncoupling.

In summary, Tan I, Tan IIA and CT restored eNOS coupling in high glucose treated EA.hy926 cells by inhibiting the activity and expression of NOX4, increasing the ratio of BH4 to BH2 and promoting the expressions of GTPCH1, DHFR, and HSP90, the effect may involve PI3K pathway. The study provides an insight into the mechanisms of endothelial protections of tanshinones. Future study on the mechanisms involved in the restoration of eNOS uncoupling by tanshinones and related compounds may help to develop new agents to treat eNOS uncoupling-mediated cardiovascular and metabolic diseases. Thus, in next part, we are going to test the effect of two new compounds derived from tanshinone on eNOS uncoupling.
7 Chapter 7. Effect of tanshinone derivatives (AC1 and AC2) on eNOS uncoupling in EA.hy926 cells

7.1 Introduction
As described in the Chapter 6, three major tanshinones, Tan I, Tan IIA and CT, are able to improve eNOS uncoupling by manipulating multiple intracellular molecular targets (eg BH4, eNOS, NOX and PI3K). This provides an inspiration to study and develop new compounds derived from tanshinones in order to improve certain feature of tanshinone, such as low bioavailability (807).

Recently, the approach that modifying the known structure and manipulating physicochemical properties to generate new compound with better ADME/Tox profile and pharmacological effect is very inspiring. Tanshinones such as Tan I, Tan IIA and CT have a wide range of beneficial actions, in particular cardiovascular protective effect. However, they exhibited a low hydrophilcity and can interact with a number of drugs. So, if we can identify compounds with similar actions as tanshinones but with a better bioavailability it will help to develop new drugs to treat eNOS uncoupling and endsothelium dysfucntions.

Thus, we will test the effect of two new tanshinone derivatives (AC1 and AC2, synthesised by the Department of Chemistry, RMIT Univeristy. Structures are not shown due to IP concerns) on eNOS uncoupling using the same model and methods as described in Chapter 6, and evaluated the potential molecular targets involved in the process.
7.2 Materials and methods

See Chapter 2, Section 2.2.1 and 2.1.6 to 2.1.13.
7.3 Results

7.3.1 Effect of AC1 and AC2 on cell viability

The toxic effect of AC1 and AC2 on EA.hy926 cells was determined by MTT assays. Unlike the previous three tanshinones, these two tanshinone derivatives did not have significant cytotoxic effect on EA.hy926 cells in the tested range of 0.1-30µmol/L up to 48 hr (Figure 7. 1), except for AC1 with a concentration of 30µmol/L after 48 hr treatment.
Figure 7.1. Cytotoxic effects of AC1 and AC2 on cultured EA.hy926 cells. Cytotoxic effects of AC1 and AC2 on ECs. EA.hy926 cells were seeded into 96-well plate at a density of 5000 cells/well. MTT assay was performed as described in Methods. Data represent the mean ± S.E. derived from five independent experiments. *, p<0.05.
7.3.2 Effect of AC1 and AC2 on high glucose induced superoxide production

As showed in Chapter 3, we observed that high glucose induced increase in superoxide production was significantly inhibited by both L-NAME (100 µM) and DPI (5 µM). We next tested the effect of AC1 and AC2 on high glucose induced superoxide production. AC1 and AC2 in a concentration range (1-10 µM) which did not affect the cell viability as determined by MTT assay, significantly and concentration-dependently inhibited high glucose induced superoxide production (Figure 7.2). Both AC1 and AC2 at 1 µM produced a similar inhibition as that caused by L-NAME at 100 µM. These results demonstrate that AC1 and AC2 have an inhibitory action on NOXs and NOS-driven superoxide production under high glucose condition.
Figure 7. 2. Effect of different concentrations of AC1 and AC2 on superoxide production in cultured EA.hy926 cells. Cells and cell homogenates were treated as described in Materials & Methods. Superoxide production was evaluated by 5 µM/L lucigenin-enhanced chemiluminescence assay. Effect of different concentrations of AC1, AC2, L-NAME (100 µM), Apo (100 µM) and DPI (5 µM) on superoxide production under high glucose condition. (##, \(p<0.01\) versus low glucose group). (##, \(p<0.01\) versus low glucose group; *, \(p<0.05\) and **, \(p<0.01\) versus 48 hr high glucose group). Data represent the mean ± S.E.M. derived from four independent experiments. -: low glucose (5.5 mM); + and ++: treatment of high glucose (35 mM) for 24 and 48 hr, respectively.
7.3.3 Effect of AC1 and AC2 on high glucose induced inhibition of NO generation

As shown in Chapter 5, high glucose significantly reduced the NO production compared to the control (5.5 mM glucose). AC1 significantly increased the NO production under high glucose condition in all tested concentrations (Figure 7.3). Likewise, AC2 exerted a similar effect on NO generation at all tested concentrations (Figure 7.3). No significant difference was observed between the action of AC1 and AC2. The effect of AC1 and AC2 was abolished by L-NAME (100 µM).
Figure 7.3. Effect of different concentrations of AC1 and AC2 on NO production in cultured EA.hy926 cells. Cells were treated as described in Methods. The intracellular level of NO was measured using the fluorescent probe DAF-2-DA. (###, $p<0.01$ versus low glucose group; **, $p<0.01$ versus 48 hr high glucose group). Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.3.4 Effect of AC1 and AC2 on NOX expression

Similar to that in Chapter 6, the expression of NOX4 was significantly increased under high glucose condition. Both AC1 and AC2 significantly inhibited the expressions of NOX4 (Figure 7.4). AC1 exerted a significant inhibitory effect on the expressions of NOX4 in a dose-dependent manner. Whereas, AC2 significantly down-regulated the expressions of NOX4 to a level similar to that at the low glucose in all tested concentrations. The results suggest that AC1 and AC2 can regulate the expression of NOX4 to exert their inhibitory effect on superoxide production under the high glucose condition.
Figure 7.4. Effects of high glucose and AC1 and AC2 on the expression of NOX4 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. A: a representative experiment showing high glucose (35 mM) induced increases in NOX4 expressions and the effects of AC1 and AC2. B: the result of densitometric analyses of graphing the expression of NOX4. (#, p<0.05 and ##, p<0.01 versus low glucose group; ^, p<0.01 and **, p<0.01 versus 24 hr and 48 hr high glucose group, respectively.). Data represent the mean ± S.E.M. derived from three independent experiments. -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.3.5 Effect of AC1 and AC2 on eNOS expression

Similarly, high glucose significantly reduced the ratio of eNOS dimer to monomer by 50% after 48 hr high glucose treatment. When the cells were treated with AC1 and AC2, the ratio of eNOS dimer to monomer was normalized. AC1 significantly increased the homodimerization of eNOS at all tested concentrations with similar efficacy (Figure 7.5). Likewise, AC2 exerted a similar effect on the homodimerization of eNOS at all tested concentrations; however, the effect of 10 µM of AC2 was weaker than that of the lower concentrations (Figure 7.5). These results indicate that AC1 and AC2 can reverse the change of eNOS from dimeric to monomeric eNOS induced by high glucose.
Figure 7.5. Effects of high glucose and AC1 and AC2 on the ratio of eNOS dimer to monomer in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The ratio of eNOS dimer to monomer decreased after high glucose treatment for 24 hr. AC1 and AC2 significantly increased the ratio of eNOS dimer to monomer which was decreased by high glucose. A: A representative experiment showing high glucose (35 mM) induced decreases in expressions of eNOS dimer, monomer and total eNOS, and the effects of AC1 and AC2. B: Results of densitometric analyses graphing the ratio of eNOS dimer to monomer. Data represent the mean ± S.E.M. derived from three independent experiments (##, $p<0.01$ versus low glucose group; *, $p<0.05$ and **, $p<0.01$ versus 48 hr high glucose group; ^, $p<0.01$ versus 24 hr high glucose group.). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.3.6 Effect of AC1 and AC2 on intracellular BH4 and BH2 levels

Similar to tanshinones, the effects of AC1 and AC2 on intracellular biopterin concentrations in high glucose treated cells were examined. The ratio of BH4 to BH2 was significantly increased by both AC1 and AC2 with concentrations of 3 and 10 µM (Figure 7. 6). The results indicate that AC1 and AC2 can reduce eNOS uncoupling by increasing the ratio of BH4 to BH2.
Figure 7.6. Intracellular levels of BH4 and BH2 in cultured EA.hy926 cells. All the samples were treated as described in Methods. The ratio of BH4 to BH2 was markedly decreased in high glucose (35 mM) condition. Tan II A reduced the high glucose induced BH4 depletion in a concentration-dependent manner. Data represent the mean ± S.E.M. derived from three independent experiments (##, *p < 0.01 versus low glucose; **, *p < 0.01 versus high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.3.7 Effect of AC1 and AC2 on expressions of GTPCH1 and DHFR

Further to determine whether AC1 and AC2 regulate biopterin concentrations via *de novo* synthesis and conversion pathways, the effects of AC1 and AC2 on the expression of GTPCH1, the rate-limiting enzyme in BH4 synthesis, and DHFR, the enzyme responsible for the conversion of BH2 to BH4, were examined. Treatment with AC1 and AC2 significantly increased the expressions of GTPCH1 and DHFR, respectively (Figure 7.7). These results indicate that AC1 and AC2 increase the ratio of BH4 to BH2 through up-regulating the expression of GTPCH1 and DHFR.
Figure 7. Effects of high glucose and AC1 and AC2 on the expressions of GTPCH1 and DHFR in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. The expressions of GTPCH1 and DHFR were inhibited by high glucose (35 mM). AC1 and AC2 (10 µM) significantly enhanced the expressions of GTPCH1 and DHFR which were suppressed by high glucose. A: a representative experiment showing high glucose (35 mM) induced decreases in expressions of GTPCH1, and the effects of AC1 and AC2. B: a representative experiment showing high glucose (35 mM) induced decreases in expressions of DHFR, and the effects of AC1 and AC2. C: Results of densitometric analyses graphing the expressions of GTPCH1 and DHFR. Data represent the mean ± S.E.M. derived from three independent experiments (#, \( p<0.05 \) and ##, \( p<0.01 \) versus low glucose group; **, \( p<0.01 \) versus high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.3.8 Effect of AC1 and AC2 on expression of HSP90

As HSP90 has an important role in regulating eNOS function and superoxide generation, the effect of AC1 and AC2 on the expression of HSP90 in the eNOS uncoupling model was also examined. As described in Chapter 5, high glucose reduced the expression of HSP90, and this reduction in expression of HSP90 were significantly reversed by AC1 and AC2 (Figure 7. 8). AC1 normalized the expression of HSP90 at all tested concentrations with similar efficacy. AC2 reversed the decrease in the expression of HSP90 at 3 and 10 µM. The results demonstrate that AC1 and AC2 can reverse the impairment of HSP90 expression elicited by high glucose exposure, resulting in improved function of eNOS.
Figure 7.8. Effects of high glucose and AC1 and AC2 on the expression of HSP90 in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. A: A representative experiment showing high glucose (35 mM) induced decreases in expressions of HSP90, and the effects of AC1 and AC2. B: Results of densitometric analyses graphing the expressions of HSP90. Data represent the mean ± S.E.M. derived from three independent experiments (#, \( p < 0.05 \) versus low glucose group; *, \( p < 0.05 \) and **, \( p < 0.01 \) versus high glucose group; ^, \( p < 0.01 \) versus 24 hr high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.3.9 Effect of AC1 and AC2 on expression of PI3K

Since NOX expression is regulated by PI3K (1043), and inhibition of PI3K resulting in decrease in the expression of NOX4 (see Chapter 5), thus, we further examined the effect of AC1 and AC2 on the expression of PI3K. AC1 and AC2 significantly inhibited the expression of PI3K at all tested concentrations showing similar efficacy (Figure 7.9). These findings indicate that PI3K pathway may be involved in the actions of AC1 and AC2 against eNOS uncoupling in EA.hy926 cells.
Figure 7.9. Effects of AC1 and AC2 on expression of PI3K in cultured EA.hy926 cells. Cells and cell lysates were treated as indicated in Methods. AC1 and AC2 significantly suppressed the expressions of PI3K which were enhanced by high glucose. A: results from a representative experiment that was performed three times with equivalent results. B: results of densitometric analyses graphing the expression of p110β. Data represent the mean ± S.E.M. derived from three independent experiments (##, p<0.01 versus low glucose group; **, p<0.01 versus high glucose group). -: low glucose; + and ++: treatment of high glucose for 24 hr and 48 hr, respectively.
7.4 Discussion

The aim of this study was to examine the effect of two new tanshinone derivatives AC1 and AC2 on endothelial function. The main finding is that high glucose induced eNOS uncoupling in human ECs can be restored by AC1 and AC2. This is the first report of the effects of AC1 and AC2 on eNOS uncoupling in human ECs.

Our findings showed that AC1 and AC2 have similar actions as tanshinones to regulate eNOS by manipulating the dimerization of eNOS rather than increase total eNOS enzymes (against the decrease in the ratio of active form of eNOS (dimer) to its inactive form (monomer)). This is consistent with their effects on BH4 levels, indicating their therapeutic effects on eNOS uncoupling as eNOS monomerization is an indicator of eNOS uncoupling (426).

It has been demonstrated that Tan I, Tan IIA and CT were capable of alleviating the oxidative stress through the inhibition of NOX4 expression (Chapter 6). In this part, we also found that AC1 and AC2 had the capability of inhibiting the expression of NOX4 which was increased in high glucose induced eNOS uncoupling cell model in EA.hy926 cell line. It is likely that AC1 and AC2 have a similar anti-oxidant action as tanshinones to inhibit ROS generating enzyme. In addition, since wortmannin significantly decreased the expression of NOX4 (Chapter 5), and AC1 and AC2 suppressed the expression of PI3K which was increased under high glucose condition, AC1 and AC2 may act by decreasing NOX4 expression and restoring eNOS uncoupling via, at least partially, PI3K signalling pathway. It has been previously reported that PI3K, PKC and NF-κB pathways are involved in the regulation on NOXs (44, 1043, 1053, 1069-1072). We have found that tanshinones suppressed the expression of PI3K under high glucose condition (Chapter 6). Further studies are needed to elucidate the role of PI3K signalling pathway in AC1 and AC2 induced regulation of NOX4 expression.

Similar to tanshinones, AC1 and AC2 were able to increase the bioavailability of BH4 under high glucose condition. This effect is likely to involve GTPCH1 and DHFR, two important enzymes involved in BH4 synthesis or conversion. The findings suggest that high glucose impaired both de novo biosynthetic and salvage
pathways of BH4, leading to intracellular BH4 deficiency and eNOS uncoupling. AC1 and AC2 may regulate BH4 synthesis to maintain adequate intracellular BH4 content by increasing the expressions of GTPCH1 and DHFR, and/or prevent BH4 from oxidation by its anti-oxidative effects. The enhancing effect of AC1 and AC2 on the ratio of BH4 to BH2 in ECs has not been reported previously. We have also observed that when the cells were incubated with high glucose initially for 24 hr then treated with wortmannin for another 24 hr, the ratio of BH4 to BH2 was increased. Taken together, these results indicate that AC1 and AC2 can restore eNOS uncoupling by increasing the ratio of BH4 to BH2 which may also involve PI3K pathway.

We have also investigated the involvement of HSP90 in actions of AC1 and AC2, AC1 and AC2 restored the decrease in the expression of HSP90 under high glucose condition. The finding indicates that AC1 and AC2 may regulate eNOS function by acting on HSP90. Similarly, it was noted that the actions of AC1 and AC2 on HSP90 expression occurred at a lower concentration that its action on BH4 levels (Figure 7. 8). Thus it is possible, AC1 and AC2 may act on multiple mechanisms on restoring eNOS uncoupling, at low concentrations it may act through regulating of interactions of HSP90 with eNOS. At a higher concentration, it may regulate expressions of GTPCH1 and DHFR and subsequent BH4 levels. Further study is necessary to elucidate the mechanism involved in AC1 and AC2’s effect on regulation of HSP90-eNOS interaction. Thus, the actions of AC1 and AC2 are similar to that observed for tanshinones (Chapter 6). Similarly, the present study does not exclude the possibility of involving other mechanisms such as S-glutathionylation of eNOS (415) in protection of AC1 and AC2 against eNOS uncoupling.

In summary, both AC1 and AC2 restored eNOS coupling by inhibiting the activity and expression of NOX4, increasing the ratio of BH4 to BH2 and promoting the expressions of GTPCH1, DHFR, and HSP90, the effect may involve PI3K pathway. The study provides an insight into the mechanisms of endothelial protections of tanshinone derivatives. Future study on the mechanisms involved in the restoration of eNOS uncoupling by AC1 and AC2 and related compounds may help to develop
new agents to treat eNOS uncoupling-mediated cardiovascular and metabolic diseases.
8 Chapter 8. General Discussion

8.1 Major finding of the study

8.1.1 Computational analysis of Danshen compounds and other natural products

Traditional drug discovery and development process involves using various in vitro and in vivo approaches to characterize the pharmacological actions and ADME/Tox profiles of compounds or drug candidates. However, the limitation of experimental capacity and chemical synthesis makes it a difficult and costly exercise to screen and test huge numbers of compounds. The development and use of in silico approaches in drug research has made it possible to virtually screen a large number of compounds without physical synthesis of these compounds. In recent years, studies using in silico approaches have been integrated into the process of drug discovery and development, which has accelerated the analysis of physicochemical properties, biological fate, toxicity and drug interactions. The application of in silico approaches has significantly enhanced the drug discovery and development process, especially for characterization of ADME/Tox profiles of various compounds or drug candidates in early stage of drug development to reduce the high cost of bench work and minimize the risk of late-stage undesired actions of drug candidates, such as undesirable ADME properties or toxicity. As many current drugs or drug candidates are derived from natural sources, predication of the biological fate and toxicity of natural products using in silico approaches in combination with in vitro and in vivo methods, will facilitate the development of novel drugs from natural compounds with reduced risks and costs.

One of the aims of the present study is to evaluate the physicochemical properties, drug-likeness and drug scores of Danshen compounds. Structures of 94 known compounds isolated from Danshen were retrieved and analysed by using relevant softwares (OSIRIS Property Explorer, ALOGPS and ACD/ChemSketch) and Ro5 principle. Among these, only 2 lipophilic compounds and 16 hydrophilic compounds showed a low drug score, indicating that most active Danshen compounds (with a confirmed pharmacological action) have the potential to be developed as a drug. In fact, Tan IIA, a major tanshinone, has been developed and used clinically in China as a drug for treating cardiovascular conditions (642). In this regard, some hydrophilic
compounds, such as danshensu, may have a high potential for being developed as a pharmaceutical drug. On the other hand, this study also revealed potential toxicity risks for the drug-like Danshen compounds. Majority of evaluated compounds showed a low toxicity risk. Only a small number of compounds possessed relevant high toxicity risks, including irritating effects for 15 lipophilic compounds (among 64 compounds tested) and mutagenicity, tumorigenicity, irritating effects for several hydrophilic compounds (5, 2, and 3 compounds respectively among 34 compounds tested). The study also identified the factors affecting the drug like property of Danshen compounds including mainly molecular weight, to a lesser extent, H-bond donor and acceptor, especially for the hydrophilic compounds. These findings may help to understand the actions of Danshen compounds in vitro and in vivo and also provide a knowledge base for designing new pharmaceutical compounds derived from Danshen compounds.

To further extend the computational study, a comprehensive search was conducted to review the current research on in silico approaches on structure-based analysis, virtual screening, drug metabolizing enzyme and drug transporters including drug interactions. A number of natural compounds have been demonstrated as substrates of P450s, UGTs, drug transporters such as P-gp, raising the potential of herb-drug interactions (Chapter 4). In silico approaches have been used successfully to study the metabolic reactions, induction, biotransformations, binding characteristics to drug metabolizing enzymes and drug transporters etc. They have also been used to predict potential toxicity of natural products and herb-drug interactions.

It should be pointed out that in silico approaches also have some challenges, such as lack of representation of the chemical entity/drug candidates or reliable experimental data for generating proper computational models. Thus, combination of in silico approaches with traditional in vitro and in vivo methods, and further developing and validating proper in silico models with less complexity, high predictability, and better correlations with available in vivo and in vitro data, are important for prediction of drug actions.
8.1.2 Experimental model of eNOS uncoupling

Increasing evidence suggests that endothelial dysfunction has been implicated in many metabolic and cardiovascular diseases resulting in significant health burden to society. Regulation of endothelial function has been proposed to be the therapeutic approach to treat relevant diseases (1073). eNOS uncoupling is a mechanism driving endothelial dysfunction. Thus, improving eNOS coupling would result in beneficial effect on endothelial function and produce favourable outcome in the treatment of metabolic and cardiovascular diseases.

One of the aims of the present study is to establish a high glucose induced eNOS uncoupling model in human endothelial cell line EA.hy926, then use it to investigate the effects of tanshinones and derivatives on eNOS uncoupling. It has been demonstrated that multiple metabolic abnormalities are associated with endothelial dysfunction with an impairment of vasodilating activity (1074-1077). Dysfunction of the endothelium is an important factor in the pathogenesis of vascular disease in diabetes (1076, 1078). Although the underlying molecular mechanism(s) by which metabolic syndrome induces endothelial dysfunction has not been fully revealed, there are many possibilities of vascular endothelial damage and increase in cardiovascular risk in these patients, in particular, hyperglycaemia. It has been reported that increased ROS production and impaired eNOS function occur under hyperglycaemia condition in vitro. Furthermore, both animal and human studies have demonstrated an association between hyperglycaemia and endothelial dysfunction (71, 1079). Endothelial dysfunction is regarded as a trigger of hyperglycaemia associated vascular disease. Therefore, a high glucose induced eNOS uncoupling model will be very useful to investigate the underlying mechanism and also examine the therapeutic effect of various compounds on endothelial function and eNOS uncoupling.

In the present study, we found that the EA.hy926 cells under high glucose condition exhibited a reduced NO production and an increased eNOS derived superoxide generation. Furthermore, we demonstrated that the posttranslational modulation of eNOS was negatively regulated under high glucose condition with reduction in the ratios of BH4 to BH2 and eNOS dimer to monomer. In addition, the expression of
GTPCH1, DHFR, and HSP90 were down regulated, whereas the expression of NOX4 and PI3K was up-regulated. These results demonstrated that the high glucose treated cell model meets the need for studying eNOS uncoupling mechanisms.

8.1.3 Effects of tanshinones and derivatives on eNOS uncoupling

Tanshinones have been reported with a variety of activities including improvement of microcirculation, dilation of coronary arteries, increase in blood flow, anti-coagulant, anti-thrombotic, anti-inflammatory, anti-angiogenesis, free radical scavenging, and mitochondria-protective effects (637, 639, 658, 659, 1080). Tan I, Tan IIA and CT are the three major active tanshinones that have been extensively studied, however, the effect on eNOS uncoupling has not been reported yet.

We have focused on the therapeutic ability of Tan I, Tan IIA and CT in endothelial dysfunction using the high glucose induced eNOS uncoupling model established. We found that high glucose induced eNOS uncoupling in human ECs can be restored by Tan I, Tan IIA and CT (Figure 8. 1). The results showed that these tanshinones increased BH4 bioavailability and NO production, reduced superoxide generation and the expression of NOX4. They also up-regulated the expressions of GTPCH1, DHFR and HSP90, and down-regulated the expression of PI3K. In addition, two synthetic analogues of tanshinones were also found with similar effects on eNOS uncoupling. The findings indicate that these tanshinones and tanshinone derivatives can restore high glucose induced eNOS uncoupling by up-regulation of BH4 bioavailability and the expression of HSP90, down regulation of NOX and PI3K pathway.
Figure 8.1. Effects of tanshinones and its derivatives on eNOS uncoupling under high glucose condition.
The study provides an insight into the mechanisms of endothelial protections of tanshinone and tanshinone derivatives. As eNOS uncoupling is a underlying mechanism of endothelial dysfunction involved in the pathogenesis of many metabolic and cardiovascular diseases, such as atherosclerosis and diabetes (76, 1081), the findings in the present study may help to develop new agents to treat eNOS uncoupling-mediated cardiovascular and metabolic diseases.
8.2 Limitations of this study

The present study has achieved the research objective to identify the effects of tanshinone and tanshinone derivatives on eNOS uncoupling and also analysed the physicochemical properties and drug potential of Danshen compounds. However, the study has some limitations.

First, we only tested the high glucose induced eNOS uncoupling. It is not clear if observed eNOS uncoupling and mechanism occur in other metabolic conditions such as hypertriglyceridemia, hyperinsulinemia and hyperglycaemia with various alterations such as increase in the level of fatty acid, small dense LDL-cholesterol, apolipoprotein B, insulin-1 growth factor, tissue Ang II, plasminogen activator inhibitor-1, C reactive protein, ROS and decrease in HDL-cholesterol (78, 1081-1084). Further studies in these conditions are necessary.

Second, the present study has used human endothelial cell line EA.hy926. Although human umbilical vein ECs are an ideal model system and widely used for cardiovascular research involving angiogenesis, arteriosclerosis, oxidative stress, tubule formation, wound healing, immune response and drug screening (1085-1087). EA.hy926 is an immortalised cell line established by fusing primary human umbilical vein cells with a thioguanine-resistant clone of A549 by exposure to polyethylene glycol (1085). There is a difference in the response to cytokines between EA.hy926 and between primary umbilical vein endothelial cells (1088, 1089). Further study using primary human endothelial cells may help to confirm the findings obtained in the present study.

Additionally, the present study only obtained preliminary evidence on mechanism of regulation of NOXs by PI3K. It has been reported that the regulation of NOXs via PI3K pathway can have several mechanisms, including protein binding with NOXs (1090), phosphorylation of NOXs (327), regulation on the recruitment of NOXs regulatory subunits (1091). Thus, to clearly identify the regulatory effect of PI3K on NOXs, more functional experiment are needed.
Finally, for the computational studies, using computational softwares and databases may help to elucidate physicochemical properties and drug potential of natural compounds, although we did not conduct biochemical experiments to validate the *in silico* results. Besides, there is a lack of reliable experimental data to generate proper computational model for further analysis of relationship between the *in vitro* and *in vivo* data. Clearly, there is a need for establishing and validating further proper models with less complexity, high predictability, and better correlations with available *in vivo* and *in vitro* data, which will have important applications in drug discovery and drug development.
8.3 Future perspectives

Our findings from this project suggest that: 1) EA.hy926 cells under high glucose exposure exhibit eNOS uncoupling through up-regulation the activity and expression of NOX4; 2) tanshinones and tanshinone derivatives reverse the high glucose induced eNOS uncoupling by improving BH4 bioavailability; 3) analysis of physicochemical properties and drug potential and in silico approaches in prediction of biological fate, toxicity and drug interactions of natural products help develop new drugs.

In future, the following research work can be carried out:

- design and synthesize new hydrophilic and lipophilic compounds.
- extend the *in vitro* study on eNOS uncoupling to investigating the effects of tanshinione compounds on eNOS uncoupling *in vivo*.
- employ gene modification technique to knock down or overexpress specific molecular target including NOXs to verify its role in ROS generation.
- conduct the functional experiment on PI3K to validate its role in regulation of NOXs. This will facilitate to elucidate the underlying molecular mechanism of regulation of eNOS uncoupling by PI3K pathway.
- carry out clinical studies. This will determine the therapeutic potential of tanshinones and tanshinone derivatives.
- incorporate biochemical experiments and computational studies. This will facilitate the study on therapeutic effect, biological fate, toxicity and drug interaction of natural compounds/products.
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