# Effects of Stilbenoid Polyphenols (Pterostilbene and Gnetol) on Cardiomyocyte Hypertrophy *in vitro* and *in vivo* in Spontaneously Hypertensive Heart Failure Rats

by

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# Abstract

Cardiac hypertrophy is increase in myocardial mass in response to stress or injury and has been identified as a potential target in the prevention of heart failure. Cardioprotective effects of resveratrol has been widely reported, however low bioavailability limits clinical use. Resveratrol, gnetol (lower bioavailability) and pterostilbene (higher bioavailability) belong to the group of stilbenoid polyphenols. Activation of AMP-activated protein kinase (AMPK), a key energy balance sensor, via phosphorylation at thr-172 is known to be protective and prevent the development of cardiac hypertrophy. Microphthalmia-associated transcription factor (MITF) is a transcription factor involved in melanogenesis. The novel role of MITF in cardiac hypertrophy has been recently identified in the literature. Here I queried first, the anti-hypertrophic effects of gnetol and pterostilbene and the involvement of AMPK and MITF *in vitro*. Secondly, I examined the cardioprotective effects and the role of bioavailabilities of resveratrol, gnetol and pterostilbene *in vivo* using spontaneously hypertensive heart failure (SHHF) rats.

Effects of gnetol and pterostilbene on ET-1-induced hypertrophy were determined: cell size by immunostaining and computer-assisted planimetry, protein synthesis measured by Click-iT® protein synthesis assay kit and fetal gene (BNP) expression by real time PCR. The role of AMPK was probed using compound C, an AMPK inhibitor and AMPKα1/2 shRNA to knockdown AMPK gene. SHHF and Sprague-Dawley control rats were treated with the stilbenoids (2.5mg/kg/day) by oral gavage. Baseline and endpoint echocardiography were performed to capture cardiac function and heart dimensions.

Gnetol inhibited all markers of hypertrophy. Pterostilbene inhibited myocyte size augmentation and protein synthesis but did not prevent fetal gene expression. Gnetol and pterostilbene activated AMPK. Indeed, pre-treatment with compound C and genetic knockdown of AMPK abolished the ability of gnetol and pterostilbene to inhibit myocyte growth. While MITF expression increased in response to ET-1, pre-treatment with stilbenoids prevented ET-1-induced MITF expression.

Gnetol and pterostilbene inhibited cardiomyocyte hypertrophy *in vitro*, at least in part, via AMPK activation. Although the stilbenoids did not prevent left ventricular hypertrophy in SHHF rat, they improved diastolic function to the same extent despite differences in their bioavailabilities. Thus, further studies on mechanisms of cardioprotective effects of stilbenoid polyphenols are warranted.

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# Dedication

*This thesis is dedicated to:*

God almighty for his sovereignty over my life

My husband and son: Johnson and Joshua Aina

My parents: Isaac and Esther Akinwumi

# Table of Contents


Abstract ..... ii

Acknowledgements ..... iii

Dedication ..... iv

Table of Contents ..... v

List of figures ..... ix

List of tables ..... x

List of abbreviations ..... xi

1.0 CHAPTER ONE: INTRODUCTION ..... 1

2.0 CHAPTER TWO: LITERATURE REVIEW ..... 6

2.1 Heart failure classification ..... 6

2.2 Diagnosis ..... 7

2.3 Epidemiology ..... 8

2.4 Etiology ..... 9

2.5 Pathogenesis and pathophysiology of heart failure ..... 10

2.5.1 Frank-Starling mechanism ..... 11

2.5.2 Renin-Angiotensin-Aldosterone system ..... 12

2.5.3 Natriuretic peptides ..... 13

2.5.4 Sympathetic nervous system ..... 14

2.5.5 Myocardial hypertrophy ..... 15

2.5.6 Fibrosis and cardiac remodelling ..... 15

2.5.7 Altered calcium homeostasis ..... 16

2.5.8 Altered bioenergetics and oxidative stress ..... 16

2.6 Hypertension and heart failure ..... 18

2.7 Drugs used in the treatment of HFrEF ..... 19

2.8 Drugs used in the treatment of HFpEF ..... 24

2.9 Cardiac hypertrophy ..... 25

2.9.1 Physiologic hypertrophic signaling ..... 27

2.9.2 Pathologic hypertrophic signaling ..... 28

2.10 AMP-activated protein kinase (AMPK) ..... 30

2.10.1 AMPK and metabolism ..... 31

2.10.2 AMPK and cardiac hypertrophy ..... 32

2.11 Microphthalmia associated transcription factor (MITF) ..... 34
2.11.1 MITF and cardiac hypertrophy ..... 35
2.11.2 AMPK and MITF cross talk ..... 37
2.12 Stilbenoids ..... 39
2.12.1 Stilbenoid structure ..... 39
2.12.2 Stilbenoid sources ..... 40
2.12.3 Bioavailability ..... 40
2.12.4 Metabolism ..... 42
2.12.5 Stability ..... 44
2.12.6 Safety ..... 45
2.13 Cardioprotective effects of stilbenoids ..... 45
2.14 Other biological effects of stilbenoids ..... 52
2.15 Animal models of hypertrophy and heart failure ..... 61
2.16 Hypothesis and objectives ..... 65
3.0 CHAPTER THREE: MATERIALS AND METHODS ..... 70
3.1 Materials ..... 70
3.2 Neonatal rat ventricular cardiomyocyte isolation and culture ..... 71
3.3 Viability tests ..... 71
3.4 Treatments ..... 72
3.5 Immunostaining and cell size measurements ..... 72
3.6 RNA extraction and polymerase chain reaction ..... 73
3.7 Protein synthesis ..... 73
3.8 Effects of stilbenoids on AMPK activation and expression ..... 74
3.9 Lentiviral preparation and infection ..... 74
3.10 Adult rat ventricular cardiomyocytes isolation and culture ..... 74
3.11 Measurements of cardiomyocyte contractility ..... 75
3.12 In vivo animal study ..... 75
3.13 Dosing and animal groups ..... 76
3.14 Body Weight measurements ..... 77
3.15 Blood pressure measurements ..... 77
3.16 Echocardiography ..... 77
3.17 Lysate preparation ..... 78
3.18 Western blotting ..... 79

3.19 Statistical analysis ..... 80
3.20 Animal ethics ..... 80
4.0 CHAPTER FOUR: ANTIHYPERTROPHIC EFFECTS OF STILBENES ..... 82
4.1 Effects of resveratrol on ET-1-induced hypertrophy ..... 82
4.2 Effects of gnetol on cardiomyocyte viability ..... 83
4.3 Gnetol inhibits hypertrophy in neonatal rat cardiomyocytes ..... 84
4.4 Effects of gnetol on adult cardiomyocyte contractility ..... 85
4.5 Effects of pterostilbene on cardiomyocyte viability ..... 86
4.6 Pterostilbene inhibits hypertrophy in neonatal rat cardiomyocytes ..... 87
5.0 CHAPTER FIVE: ANTIHYPERTROPHIC SIGNALING MECHANISMS ..... 89
5.1 Gnetol activates AMPK via phosphorylation ..... 89
5.2 Inhibition of AMPK abolishes the anti-hypertrophic effects of gnetol ..... 91
5.3 Genetic knockdown of AMPK abolishes the anti-hypertrophic effects of gnetol ..... 92
5.4 Pterostilbene activates AMPK via phosphorylation ..... 93
5.5 Inhibition of AMPK abolishes anti-hypertrophic effects of pterostilbene ..... 95
5.6 Genetic knockdown of AMPK abolishes the anti-hypertrophic effects of pterostilbene ..... 96
5.6 AMPK knockdown efficiency ..... 97
5.7 Pharmacological inhibition of MITF blunts hypertrophic response to ET-1 ..... 99
5.8 ET-1 increases MITF expression ..... 100
5.9 Effects of stilbenes on MITF expression ..... 101
5.10 Stilbenoids prevented the increase in MITF expression induced by ET-1 ..... 103
5.11 Pharmacological inhibition of AMPK blunts MITF expression ..... 104
5.12 Effect of compound C on MITF expression with and without ET-1 ..... 105
6.0 CHAPTER SIX: EFFECTS OF STILBENOIDS ON CARDIAC STRUCTURE AND FUNCTION IN SPONTANEOUSLY HYPERTENSIVE HEART FAILURE RATS ..... 107
6.1 Cardiac structure parameters ..... 107
6.1.1 Left ventricular mass and body weights (normalized with tibia length) ..... 107
6.1.2 Treatment groups ..... 109
6.1.3 Endpoint intraventricular septal thickness during systole and diastole ..... 110
6.1.4 Baseline intraventricular septal thickness during systole ..... 112
6.2 Cardiac function parameters ..... 113
6.2.1 Systolic parameters: ejection fraction and fractional shortening ..... 113
6.2.2 Diastolic parameter: iso-volumetric relaxation time ..... 115

6.2.3 Cardiac function - blood pressure ..... 116
6.3 Hypertrophy signaling results ..... 118
6.3.1 Levels of AMPK in heart tissues ..... 118
6.4 Fibrosis markers ..... 120
6.4.1 Smooth Muscle Actin (SMA) levels in heart tissue ..... 120
6.4.2 Collagen I levels in heart tissue ..... 121
7.0 CHAPTER SEVEN: DISCUSSION ..... 123
7.1 Anti-hypertrophic effects of stilbenoids in vitro ..... 123
7.2 Anti-hypertrophic signaling of stilbenoids in vitro ..... 125
7.2.1 AMPK signaling ..... 125
7.2.2 MITF signaling ..... 127
7.3 AMPK – MITF crosstalk ..... 128
7.4 Effects of stilbenoids on hypertrophy in vivo ..... 129
7.5 Anti-hypertrophic signaling in vivo ..... 133
7.6 Anti-fibrotic signaling in vivo ..... 135
7.7 Bioavailability and bioactivity of stilbenoids ..... 138
8.0 CHAPTER EIGHT: CONCLUSION AND FUTURE DIRECTIONS ..... 142
Creative common licensed materials ..... 144
REFERENCES ..... 145

# List of figures

Figure 1: Path to functional decompensation in heart failure ..... 11
Figure 2: Frank-Starling curve ..... 12
Figure 3 Chemical structures of selected stilbenoids showing common stilbene backbone ..... 39
Figure 4: Metabolic pathways and common metabolites of pterostilbene ..... 43
Figure 5: Metabolic pathways and common metabolites of resveratrol ..... 44
Figure 6: Proposed AMPK-MITF signaling in cardiomyocytes ..... 67
Figure 7: Treatments and study timeline ..... 76
Figure 8: Effect of resveratrol on ET-1 induced hypertrophy ..... 82
Figure 9: Effect of gnetol on cardiomyocyte viability ..... 83
Figure 10: Effect of gnetol on the three markers of hypertrophy ..... 84
Figure 11: Effect of gnetol on adult cardiomyocyte contractility ..... 85
Figure 12: Effect of pterostilbene on cardiomyocyte viability ..... 86
Figure 13: Effects of pterostilbene on three markers of hypertrophy ..... 87
Figure 14: Effect of gnetol on AMPK activation (total AMPK and phosphorylated AMPK) ..... 90
Figure 15: Effect of compound C, a pharmacological inhibitor of AMPK, on anti-hypertrophic effects of gnetol ..... 91
Figure 16: Effect of shRNA silencing of AMPK on the anti-hypertrophic effect of gnetol ..... 92
Figure 17: Effect of pterostilbene on AMPK activation (total AMPK and phosphorylated AMPK) ..... 94
Figure 18: Effect of compound C, a pharmacological inhibitor of AMPK on anti-hypertrophic effect of pterostilbene ..... 95
Figure 19: Effect of shRNA silencing on anti-hypertrophic effect of pterostilbene ..... 96
Figure 20: shRNA silencing of AMPKα isoforms ..... 98
Figure 21: Effect of the MITF inhibitor, ML329, on ET-1 induced hypertrophy ..... 99
Figure 22: Effect of ET-1 on MITF expression ..... 100
Figure 23: Effects of gnetol and pterostilbene on MITF expression ..... 102
Figure 24: Effects of stilbenoid pre-treatment on MITF expression with and without ET-1 ..... 103
Figure 25: Effect of compound C and stilbenoid pretreatment on MITF expression induced by ET-1 ..... 104
Figure 26: Effect of compound C on ET-1 induced MITF expression ..... 105
Figure 27: Effects of stilbenoids on left ventricular mass and body weights ..... 108
Figure 28: Animal and treatment groups ..... 109
Figure 29: Effects of stilbenoids on intraventricular septal thickness during systole and diastole ..... 111
Figure 30: Baseline intraventricular septal thickness during systole ..... 112
Figure 31: Effects of stilbenoids on systolic parameters ..... 114
Figure 32: Effects of stilbenoids on diastolic function- isovolumetric relaxation time ..... 115
Figure 33: Effects of stilbenoids on systolic blood pressure over time ..... 117
Figure 34: Levels of AMPK activation in heart tissues with and without stilbenoid treatment ..... 119
Figure 35: Levels of SMA in heart tissues with or without stilbenoid treatment ..... 120
Figure 36: Levels of collagen I in heart tissue with and without stilbenoid treatment ..... 121

# List of tables

Table 1: Half-lives and oral bioavailabilities of stilbenoids after oral administration in rats ..... 41
Table 2: Antibody list: sources, dilutions and incubation conditions ..... 80
Table 3: Effect of stilbenoids on blood pressure ..... 116

# List of abbreviations

| Abbreviation | Full text |
|---|---|
| ACC | acetylCoA carboxylase |
| ACC/AHA | American college of cardiology/ American heart association |
| ACE | angiotensin converting enzyme |
| ACEI | angiotensin converting enzyme inhibitors |
| AGE | advance glycation end product |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleotide |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| Ang II | angiotensin II |
| ANP | atrial natriuretic peptide |
| ARB | angiotensin receptor blocker |
| AT-1 receptor | angiotensin II receptor type-1 |
| ATP | adenosine triphosphate |
| BCL2 | B-cell lymphoma 2 |
| BNP | brain natriuretic peptide |
| CAMKKβ | calcium/calmodulin-dependent protein kinase kinase beta |
| cAMP | cyclic adenosine monophosphate |
| CCS | Canadian cardiovascular society |
| CDK2 | cyclin-dependent kinase |
| CHD | coronary heart disease |
| COX | cyclooxygenase |
| CPT-1 | carnitine palmitoyl transferase-1 |
| CREB | cAMP response element-binding protein |
| CVD | cardiovascular disease |
| CYP | cytochrome P450 |
| DAG | diacylglycerol |
| eEF2 | eukaryotic elongation factor 2 |
| EDD | end-diastolic dimension |
| eNOS | endothelial nitric oxide synthase |
| Erbb2 | erb-b2 receptor tyrosine kinase 2 |
| Erbin | Erbb2-interacting protein |
| ERK | extracellular signal-regulated protein kinase |
| ET-1 | endothelin-1 |
| ESD | end-systolic dimension |
| GDP | guanosine diphosphate |
| GLUT4 | glucose transporter type 4 |
| GPCR | G-protein coupled receptor |
| GSK-3β | glycogen synthase kinase-3-beta |
| GST | glutathione-S-transferase |
| GTP | guanosine triphosphate |
| HBA1c | glycated hemoglobin |

| | |
|---|---|
| HDL | high density lipoprotein |
| HFpEF | heart failure with preserved ejection fraction |
| HFrEF | heart failure with reduced ejection fraction |
| HLH | helix-loop-helix |
| HO-1 | heme oxygenase-1 |
| HOMA | homeostatic model of assessment of insulin resistance |
| IGF-1 | insulin-like growth factor-1 |
| IP3 | Inositol-1,4,5-triphosphate |
| IVRT | iso-volumetric relaxation time |
| IVSd | intraventricular septal thickness during diastole |
| IVSs | intraventricular septal thickness during systole |
| LDL | low density lipoprotein |
| L-DOPA | L-3,4-dihydrophenylalanine |
| LKB | liver kinase B |
| MAPK | mitogen-activated protein kinase |
| MDA | malondialdehyde |
| MEK | mitogen-activated protein kinase kinase |
| MHC | myosin heavy chain |
| MITF | Microphthalmia-associated transcription factor |
| MLC-1a | myosin light chain-1a |
| mPTP | membrane permeability transition pore |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| mTOR | mammalian target of rapamycin |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NE | norepinephrine |
| NFκB | nuclear factor kappaB |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| NrF2 | nuclear factor erythroid 2-related factor |
| NYHA | New York heart association |
| P70S6K | ribosomal protein S6 kinase |
| PAI-1 | plasmogen activator inhibitor-1 |
| PFK-2 | phosphofructokinase-1 |
| PGC-1α | peroxisome proliferator-activated receptor gamma-coactivator 1-alpha |
| PI3K | phosphatidylinositol-3-kinase |
| PIP2 | phosphatidylinositol-4,5-biphosphate |
| RAAS | renin-angiotensin-aldosterone system |
| ROS | reactive oxygen species |
| SD | Sprague-Dawley |
| Ser | serine |
| SERCA | sarcoplasmic-endoplasmic reticulum calcium ATPase |
| SHHF | spontaneously hypertensive heart failure-prone |
| SHR | spontaneously hypertensive rats |
| shRNA | short hairpin RNA |
| SMA | smooth muscle actin |

| | |
|---|---|
| SOD | superoxide dismutase |
| SULT | sulphotransferase |
| TAC | transverse aortic banding |
| TBX2 | T-box transcription factor-2 |
| TGFβ | transforming growth factor-beta |
| Thr | threonine |
| TNFα | tumor necrosis factor alpha |
| TRYP | tyrosinase related protein |
| TSC | tuberous sclerosis |
| UCP-1 | uncoupling protein 1 |
| UDP | uridine-5-diphospho |
| UGT | UDP-glucuronosyltransferase |
| WHO | world health organization |
| WKY | Wistar-Kyoto |
| α-MSH | alpha-melanocyte stimulating hormone |

# 1.0 CHAPTER ONE: INTRODUCTION

According to the World Health Organization, cardiovascular disease is the foremost cause of death worldwide.[1] In Canada, heart disease is the second leading cause of death, next to cancer.[2] Among different groups of cardiovascular disorders, a trend of steadily increasing prevalence of heart failure has been reported over the past few decades.[3] For instance, in the United States alone, the number of people living with heart failure as of 2014 was 6.5 million. This number is projected to reach 8 million by the year 2030.[4] In Canada, the number of people currently living with heart failure is about 600,000 and up to 50,000 new cases are recorded every year.[5] Despite the currently available options for management, mortality rates remain high. About 50% of people diagnosed with heart failure will die within five years and 28% die within one year.[6] Possible contributing factors to the increased prevalence of heart failure include an aging population, as well as advanced medical care for the treatment and management of other cardiovascular diseases such as hypertension, myocardial infarction and stroke.[7] Since more patients survive myocardial infarction and stroke, more patients in turn are at risk for the development of heart failure.

Considering the steadily rising costs of treatment, hospitalization and end of life care,[7] extensive research is required to understand underlying mechanisms that may be exploited to prevent heart failure progression. Common risk factors for heart failure include hypertension, previous myocardial infarction, coronary artery disease and diabetes.[8] One major convergence point of these risk factors is the development of cardiac hypertrophy. Cardiac hypertrophy is the enlargement of heart muscle that occurs in response to injury or hemodynamic stress, such as myocardial infarction or uncontrolled hypertension.[9] Although hypertrophy was earlier thought to be required for compensation,[10] it is now well established that hypertrophy leads to functional

decompensation.[11-13] Thus, prevention of left ventricular hypertrophy has been identified as a critical therapeutic strategy that may be exploited to prevent heart failure.[14]

Cardiac hypertrophy can be classified as pathologic or physiologic. Pathologic hypertrophy is enlargement of myocardial mass induced by injury or stress to the myocardium, while physiologic hypertrophy occurs when increased myocardial mass is induced by increased energy demands such as in athletes and during pregnancy.[15] The underlying signaling pathways differ during physiologic versus pathologic hypertrophy.[15] Cardiac hypertrophy can also be classified as concentric or eccentric hypertrophy which may be due to pressure or volume overload, respectively.[16] Examples of pressure overload include hypertension and aortic stenosis. Consistently high end-systolic pressures cause addition of sarcomeres in parallel which leads to concentric hypertrophy, characterized by increased ventricular wall thickness with little or no change in the chamber volume.[17] Conversely, volume overload may result from mitral valve regurgitation or systolic dysfunction, in which case blood pools in the heart and causes ventricular dilation.[18] Volume overload results in eccentric hypertrophy, where sarcomeres are added in series and chamber volume increases with little or no change in ventricular wall thickness.

Evidence in the literature suggests that prevention of cardiac hypertrophy or cardiac remodeling, for instance, with the use of drugs targeting the renin-angiotensin system, leads to better outcomes in heart failure patients.[19, 20] Studies in various animal models have also shown that prevention of cardiac hypertrophy is particularly beneficial to heart function.[21, 22] Current heart failure treatment options focus on reducing the workload of the heart and inhibiting the renin-angiotensin-aldosterone system. Many Canadians are turning to natural products to supplement conventional treatment options. 56 % of Canadians use natural health products at least once per week.[23] Prevention of cardiac hypertrophy may also be achieved using naturally occurring molecules that

act through other signaling pathways. For example, naturally occurring polyphenols such as resveratrol prevent the development of cardiomyocyte hypertrophy *in vitro* and also protect heart function in animal models of heart disease.[24-26] Thus, in this research project I set out to determine the effects of two resveratrol analogues (pterostilbene and gnetol) on cardiomyocyte hypertrophy *in vitro* and also in the spontaneously hypertensive heart failure (SHHF) rat model, which is an animal model of hypertension and cardiac hypertrophy progressing to heart failure.

Resveratrol is a stilbenoid polyphenol that has been widely studied for its cardioprotective effects among other beneficial biological effects.[27] However, resveratrol has poor bioavailability, and it is rapidly metabolized by first pass metabolism.[28] This greatly limits the amounts of free resveratrol that reaches the systemic circulation. The effect of bioavailability of stilbenoid polyphenols on their bioactivities is not well understood. Thus, I selected structural analogues of resveratrol such as pterostilbene which has a much higher bioavailability[29] and gnetol, which has also been reportedly used in south-east Asian traditional medicine but with lower bioavailability.[30] Despite wide and extensive research on the cardioprotective effects of resveratrol, the effects of other stilbenoids have not been carefully explored. Specifically, the effects of pterostilbene and gnetol on cardiomyocyte or cardiac hypertrophy have not been previously studied. Moreover, the effects of resveratrol on cardiac function in the SHHF rat model have not been examined.

Therefore, I predicted that similar to resveratrol, pterostilbene and gnetol would exert anti-hypertrophic effects in isolated cardiomyocytes due to similarities in chemical structures and possibly common mechanisms of action. Furthermore, a comparative study of cardioprotective effects of resveratrol, pterostilbene and gnetol was performed *in vivo* using the same dose for the three compounds. Pterostilbene with higher bioavailability may exert higher biological activity and gnetol, despite its lower bioavailability may exert similar biological effect compared to

resveratrol. Results of this comparative study delineate the effects of stilbenoid polyphenols on cardiac function in the SHHF rat model and also on the significance of high or low bioavailabilities of stilbenoid polyphenols.

# CHAPTER 2

# LITEREATURE REVIEW

# 2.0 CHAPTER TWO: LITERATURE REVIEW

## 2.1 Heart failure classification

Heart failure is a progressive clinical syndrome characterized by reduced ability of the heart to fill and pump blood efficiently to meet the metabolic demands of the body. Common signs and symptoms of heart failure include fatigue, dyspnea and fluid retention causing swelling of the ankles and legs.[31] There are different ways to classify heart failure. According to the American College of Cardiology and American Heart Association (ACC/AHA), heart failure usually begins with predisposing factors, gradually progresses to structural disease with or without symptoms, and finally results in the refractive stage.[32] The ACC/AHA classification thus stratifies heart failure into stages A, B, C and D based on the presence or absence of risk factors and progression of disease. The New York Heart Association (NYHA) classifies heart failure into classes I, II, III, and IV based on the functional capabilities of the patient and the clinician's subjective evaluation.[33]

Heart failure may be associated with systolic and/or diastolic dysfunction. Systolic and diastolic dysfunctions occur when there is a problem with contraction and relaxation, respectively. Heart failure is also classified based on ejection fraction, which is the percentage of blood ejected per heartbeat. Heart failure with reduced ejection fraction (HFrEF) is usually associated with systolic and/or diastolic dysfunction, while heart failure with preserved ejection fraction (HFpEF) is mainly associated with diastolic dysfunction.[34] The definition of ejection fraction cut off for this classification varies by guidelines. For example, the ejection fraction cut off within the Canadian Cardiovascular Society (CCS)[31] and ACC/AHA guidelines[35] is 40% while the cut off value set by Heart Failure and Echocardiography Associations of the European Society of Cardiology is 50%.[36] Although most heart failure studies are focussed on HFrEF, emerging evidence now reveal that HFpEF has similar prevalence and mortality rates and thus should attract comparable attention.[37]

In a study by Owan *et al.*, 53% of hospitalized heart failure patients had reduced ejection fraction while the remaining 47% had preserved ejection fraction.[38] Furthermore, a recent systematic review showed that HFpEF is more prevalent than HFrEF in patients older than 60 years.[39] Patients with HFpEF exhibit signs and symptoms of heart failure despite normal ejection fraction and ventricular volumes.[37]

Congestive heart failure refers to a form of heart failure with marked signs of fluid retention in the systemic and/or pulmonary circulation. The use of this term has been limited because many heart failure patients do not exhibit these congestive symptoms.[31] Thus a broader term, "heart failure," is used to encompass patients with and without congestive signs and symptoms. Another commonly used term, acute decompensated heart failure, refers to sudden worsening of heart failure symptoms that require urgent care and hospitalization.[40]

## 2.2 Diagnosis

Diagnosis of heart failure is established following thorough medical history review, physical evaluation, electrocardiogram, chest X-ray and blood tests to detect specific markers of ventricular dysfunction and myocyte cell death such as brain natriuretic peptides (BNP), N-terminal pro-BNP and cardiac troponin C. Transthoracic echocardiography is also recommended to determine the hemodynamic status of the heart.[31] Echocardiography is used to measure blood flow in and out of the heart and estimate ejection fraction. BNP is a natriuretic peptide produced in the ventricles in heart failure patients upon stretching of the myocardium. Measurement of plasma levels of BNP is useful to confirm or rule out heart failure particularly when the clinical diagnosis is uncertain.[41] High levels of troponin C are detectable in the blood 3-4 h after myocardial infarction and indicate myocardial damage. Troponin C is a contractile protein expressed only in myocytes and is released into the systemic circulation upon myocyte loss. Diagnosis of HFpEF may be more difficult due

to the presence of co-morbidities and other possible differential diagnoses. HFpEF patients may present with normal BNP and troponin levels. Thus, new myocardial remodelling markers such as matrix metalloproteases have been recommended.[42, 43]

## 2.3 Epidemiology

The epidemiology of heart failure is dynamic and has evolved over the past few decades. Between 1971 and 1993, there was a marked increase in hospitalization rates for heart failure in the United States particularly within the age group of 65 years or older.[44] However, recent studies have indicated that the incidence of heart failure declined between 1997 and 2007 in a population-based study of patients in Ontario, Canada.[45] While incidence may have declined or remained stable in recent times, prevalence and cost of heart failure treatment are increasing steadily.[3, 46] This, arguably, may be due to improved survival and management strategies for heart failure-predisposing factors coupled with an aging population.[7] Furthermore, prevalence and associated costs of heart failure are projected to increase further over the next several years.[7, 46]

In Canada, the number of people living with heart failure is about 600,000 and up to 50,000 new cases are recorded every year.[5] In the United States, the estimate of patients living with heart failure increased from 5.7 million in 2012 to 6.5 million in 2014 and is projected to reach 8 million people by 2030.[4] Heart failure has a high hospitalization and readmission rate with a poor prognosis and even greater prevalence among the elderly above the age of 65.[4, 31] Despite the currently available management options, mortality rates remain high. About 50% of those diagnosed with heart failure will die within five years and 28% die within one year.[6] Incidence is higher among males than in females and also higher among black Americans compared to their white counterparts.[44] Considering the steadily rising costs of treatment, hospitalization and end of life care,[7] extensive

research is required to understand underlying mechanisms that may be manipulated to prevent heart failure progression.

## 2.4 Etiology

The leading causes of heart failure are hypertension, coronary heart disease and diabetes.[8] Other causes include atrial fibrillation, valvular heart disease and obesity.[8] One key convergence point for the major heart failure risk factors is the development of cardiac hypertrophy.[11, 13, 47] Adequate blood pressure control reduces the risk of ventricular hypertrophy and is thought to delay the development of heart failure. In a clinical study focussed on patients with high risk of heart failure, an antihypertensive, ramipril, reduced new cases of heart failure occurrences.[48] This study also identified the most prominent risk factors for heart failure to be coronary artery disease, microalbuminuria, left ventricular hypertrophy, advanced age and diabetes.[48] In addition to lowering blood pressure, regression of ventricular hypertrophy and remodelling confers greater benefits in cardiovascular diseases.[49-51] For example, in the Losartan Intervention For Endpoint Reduction in Hypertension (LIFE) study, the regression of left ventricular hypertrophy during treatment was associated with lower risks of cardiovascular mortality and morbidity, independent of blood pressure-lowering effects.[52]

Another study indicated that hypertension precipitated hospitalization in patients with previous myocardial infarction but with no history of heart failure.[53] In the Trandolapril Cardiac Evaluation (TRACE) trial, 54.5% of myocardial infarction survivors developed heart failure after discharge.[54] Thus myocardial infarction is a critical risk factor in the development of heart failure. Furthermore, the Framingham study established a link between heart failure and diabetes partly due to the development of diabetic cardiomyopathy. In this study, diabetic men had a 3.8-fold increased risk of developing heart failure, while diabetic women had a 5.5-fold higher relative risk.[55]

## 2.5 Pathogenesis and pathophysiology of heart failure

The fundamental feature of heart failure is the inability of the heart to fill with, and efficiently eject, blood resulting in reduced cardiac output. Partial or total occlusion of blood flow to the heart muscle causes myocardial infarction which then results in loss of myocytes, impaired contractility and poor electrical conduction in the heart.[56] Further myocyte loss may occur through necrosis or apoptosis in a failing heart.[57, 58] Changes in expression of contractile proteins and calcium-handling proteins may also alter contractility.[59] Moreover, hemodynamic stress such as pressure or volume overload causes cardiac hypertrophy and remodeling.

When cardiac output is reduced below a certain level, baroreceptors detect poor perfusion of organs and activate compensatory mechanisms to increase cardiac output.[60] Neuro-hormonal compensatory mechanisms in heart failure include activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS), which help to augment cardiac output in the short term.[61] However, when these compensatory mechanisms become chronically activated, they are no longer able to maintain functionality. Figure 1 shows the path to functional decompensation in heart failure with specific examples.

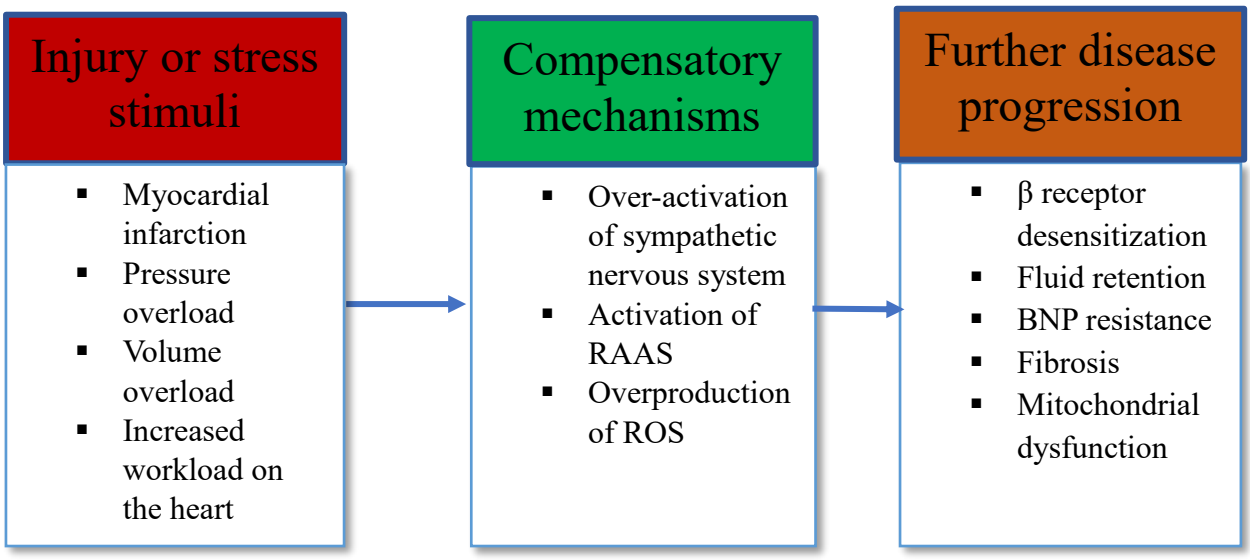



**Figure 1: Path to functional decompensation in heart failure**

It is important to note that apart from the damage to the heart by initial injury or stress stimuli, chronic activation of compensatory mechanisms also contributes to further damage and disease progression.[62]

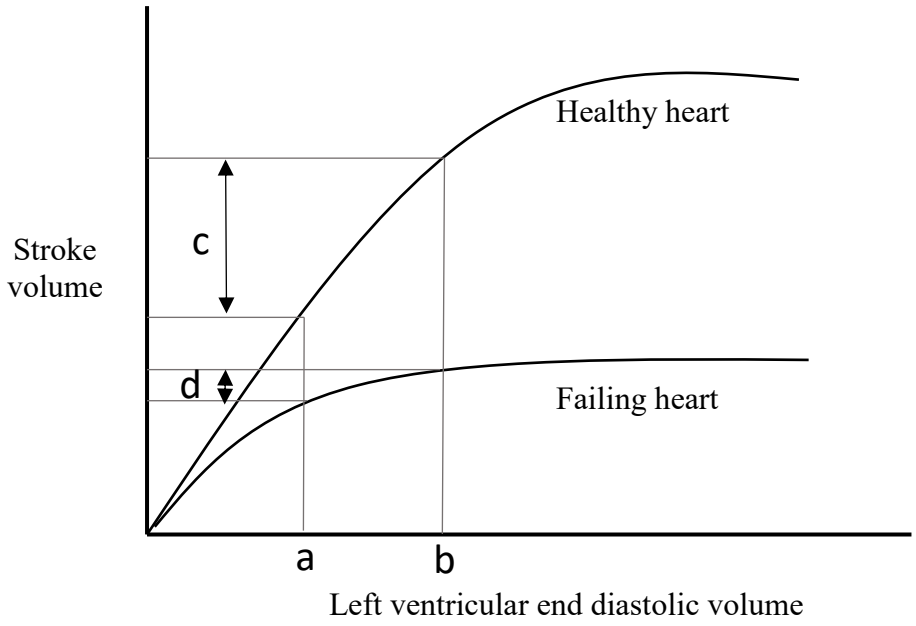
### 2.5.1 Frank-Starling mechanism

According to the Frank-Starling mechanism, stretching increases myocyte length and thus results in greater force of contraction and increased stroke volume (Figure 2).[63] This increase in myocyte length can be achieved by increasing end diastolic volume (i.e. preload). In a study done in rat ventricular muscle, stretching of the heart muscle increased sarcomere lengths. This was associated with increased calcium sensitivity, which caused more efficient force generation during interaction between actin and myosin filaments.[64] The force-length relationship may be explained by reduced lattice spacing of myofilaments, which increases the probability of calcium-dependent cross-bridge formation.[64]

In a healthy heart, increased preload results in increased stroke volume, while contractility and afterload remain constant. In a heart failure model induced by chronic coronary

microembolization, the Frank-Starling mechanism was induced in the early phase of heart failure development. [65] However, as the disease progressed, further increase in preload resulted in reduced contractility and consequently reduced stroke volume.[65] Inability of the failing heart to respond to increased preload may be due, in part, to impaired calcium handling[66, 67] and/or possible disruption in heart structure due to sarcomere disarray[65] that occur during heart failure development.

**Figure 2: Frank-Starling curve**




Increase in left ventricular end-diastolic volume from point (a) to (b) results in a dramatic increase in stroke volume (c) on the healthy heart curve but only a modest increase (d) on the failing heart curve.

### 2.5.2 Renin-Angiotensin-Aldosterone system

Reduced renal perfusion in the juxtaglomerular apparatus stimulates the production of renin in the kidney.[68] Renin catalyzes the conversion of angiotensinogen to angiotensin (Ang) I, which is then converted to Ang II by angiotensin converting enzyme (ACE).[69] Ang II increases cardiac output by causing vasoconstriction thus increasing total peripheral resistance. In addition, there is increased aldosterone secretion from the adrenal cortex, which enhances water and sodium

retention.[70] Furthermore, high Ang II levels promote secretion of antidiuretic hormone (vasopressin) from the pituitary gland.[71] Reduced cardiac output, detected by arterial baroreceptors, may also directly induce secretion of vasopressin.[72] Vasopressin binds to the Vasopressin V1b receptor, also known as antidiuretic hormone receptor 1b, and causes water retention in the distal collecting duct thereby increasing preload.

The overall effect of activation of the RAAS is to increase venous return to the heart and increase salt and water retention which increases preload. In the short term, increased preload leads to increased force of contraction and consequently increased cardiac output. However, when preload is increased beyond the linear range of the Frank-Starling curve, decompensation occurs as further preload increase does not result in increased force of contraction.[63]

### 2.5.3 Natriuretic peptides

Natriuretic peptides are a group of endogenous peptides that act by counteracting the effects of RAAS activation to maintain fluid homeostasis.[73] While activation of RAAS leads to sodium and water retention and vasoconstriction, the natriuretic peptides induce the opposite, sodium and water excretion and vasodilation.[74] Of the 6 natriuretic peptides identified, atrial natriuretic peptide (ANP) and BNP are the most important for fluid and blood pressure homeostasis.[75] ANP is produced in the atria while BNP is produced in the ventricles, although originally isolated from porcine brain.[76] Production of ANP and BNP is stimulated by volume overload that causes stretching of the atrial and ventricular walls.[77] In normal physiology, natriuretic peptides are able to counteract the effects of RAAS over-activation. However, this is not so during heart failure. Rather, excessive fluid retention leads to excessive production of BNP and ANP in heart failure patients. The resulting fluid overload exceeds the capacity of BNP to clear the fluids. Plasma BNP level is particularly high in heart failure patients and thus used as a clinical diagnostic biomarker.[78]

Natriuretic peptides act by interacting with natriuretic peptide receptors, NPR-A and NPR-B. These are guanylyl cyclase-linked transmembrane receptors.[79] A third natriuretic receptor, NPR-C, functions as a clearance receptor, and acts as the major clearance route for natriuretic peptides.[80] Alternatively, natriuretic peptides can be degraded by enzymes such as neprilysin, a neutral endopeptidase which is a target of a newer heart failure therapy, Entresto.[81] The inability of natriuretic peptides to maintain homeostasis in heart failure patients is partly due to natriuretic peptide receptor desensitization or deficiency.[82]

### 2.5.4 Sympathetic nervous system

Activation of the sympathetic nervous system occurs when baroreceptors in the carotid sinus and aortic arch detect low arterial pressures due to low cardiac output.[60] Epinephrine, norepinephrine (NE) and vasopressin are released, thus increasing sympathetic activity in the heart and the peripheral circulation. This leads to increased heart rate, increased contractility, venous and arterial vasoconstriction and increased venous return.[70] Activation of the sympathetic nervous system also augments stroke volume by increasing intracellular calcium during contraction leading to greater contractile force.[83] The sympathetic nervous system is over-activated in heart failure.[84, 85] Therefore $\beta_1$-adrenergic receptors are desensitized leading to impaired inotropic response.[86, 87] Furthermore, excessively high heart rate increases metabolic demands on the heart and worsens the existing myocardial dysfunction.

$\beta_1$-adrenergic receptor desensitization is an autoregulatory process that prevent over-activation of receptors.[88] The mechanisms underlying $\beta_1$-adrenergic receptor desensitization involve initial uncoupling of receptors from adenylyl cyclase, then internalization of the uncoupled receptor followed by phosphorylation of internalized receptors. Phosphorylation may occur at multiple sites

resulting in binding of β-arrestin with receptors, which leads to termination of adrenergic receptor activity.[88]

### 2.5.5 Myocardial hypertrophy

High systolic pressures and incomplete voiding of the ventricles during heart failure increase chamber wall stress.[63] According to the Laplace law, wall stress is directly proportional to ventricular pressure and chamber radius, and inversely proportional to ventricular wall thickness.[89] In response to sustained pressure and chamber dilatation, ventricular myocytes undergo hypertrophy to maintain contractile force and withstand high wall stress.[89] Increased ventricular wall thickness maintains cardiac output in the short term. However, with prolonged and sustained hypertrophy, the left ventricular chamber becomes excessively dilated and unable to contract properly. Apart from their direct pro-hypertrophic effects on cardiomyocytes, Ang II and epinephrine may also stimulate cardiomyocyte hypertrophy indirectly by increasing blood pressure.[90]

### 2.5.6 Fibrosis and cardiac remodelling

In heart failure with diastolic dysfunction, fibrosis increases ventricular wall stiffness. Thus the ventricles are unable to relax and fill properly.[91] Cardiac fibrosis, which occurs as a reparative response to injury or stress, begins with increased collagen deposition in the extracellular matrix.[92] Moreover, activation of myofibroblasts, production of cytokines and recruitment of inflammatory cells further impair the integrity of the myocardium.[93] Chronic activation of RAAS may also activate the development of fibrosis. In neonatal rats for example, Ang II stimulates the activation and proliferation of cardiac fibroblasts.[94] Cardiac remodelling is a broader term that encompasses structural changes in heart size, geometry shape and function that occur due to cardiac injury such as myocardial infarction.[95] Changes in heart geometry include ventricular dilatation, characterized

by lengthening and thinning of the infarct area.[96] The shape of the heart also changes from elliptical to spherical, and this may affect normal cardiac function via alterations in ventricular torsion and rotation during systole and diastole.[97]

### 2.5.7 Altered calcium homeostasis

Significant changes in calcium homeostasis also contribute to initiation and progression of heart failure.[98] During contraction calcium is released from the sarcoplasmic reticulum into the cytosol via a calcium-triggered calcium release process.[99] For relaxation to occur, the released calcium must be taken up back into the sarcoplasmic reticulum through the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA), while a small portion is pumped out of the cell via the sodium-calcium exchanger on the sarcolemma.[100]

Calcium release and reuptake occur in a finely co-ordinated and rhythmic fashion that corresponds to contraction and relaxation respectively.[99] This is known as calcium handling. Optimal calcium handling is important and required for efficient contraction and relaxation. Any factor that causes inability of the heart to recycle calcium efficiently may result in systolic and/or diastolic function. For example, a failing heart is characterized by high diastolic cytosolic calcium which may be due to incomplete re-uptake by SERCA[101] or as a result of leakage through ryanodine receptors.[102] Expression and activity of SERCA2a is reduced and has been correlated to the level of dysfunction in heart failure.[103] Restoration of SERCA2a activity via gene transfer improved contractility in human myocardium.[104] Thus modulation of calcium handling abilities of cardiomyocytes may be exploited for future heart failure therapies.

### 2.5.8 Altered bioenergetics and oxidative stress

Bioenergetics refers to the ability of a cell or system to generate its own energy. The heart requires a lot of energy to function and thus consists of huge mitochondrial mass. In fact, about 40 percent

of cardiomyocyte volume is occupied by mitochondria.[105] The mitochondrion is the power house of every cell, where energy is generated as adenosine triphosphate (ATP) via oxidative phosphorylation in the electron transport chain. However, the process of energy generation is also accompanied by generation of reactive oxygen species (ROS). Production of a limited amount of ROS is normal and this can be easily cleared by endogenous antioxidant defence enzymes such as manganese superoxide dismutase, catalase and glutathione peroxidase. Inefficient transfer of oxygen in the electron transport chain may lead to overproduction of ROS that overwhelms these antioxidant enzymes and thus leads to oxidative stress.[106] In some cases, inadequate levels of endogenous antioxidant enzymes such as manganese superoxide dismutase may contribute to the development of heart failure.[107, 108]

Oxidative stress has been identified as one of the hallmarks of heart failure pathophysiology.[109] This is characterized by overproduction of ROS and deficiency in the innate antioxidant enzymes. ROS damages cardiomyocytes via lipid peroxidation, protein oxidation and DNA oxidation. Furthermore, altered mitochondrial biogenesis, the ability of mitochondria to regenerate, limits the energy-generating capacity of cardiomyocytes in heart failure.[110] This involves downregulation of genes that modulate energy metabolism and mitochondrial biogenesis such as peroxisome proliferator-activated receptor alpha (PPAR-α) and peroxisome proliferator-activated receptor gamma co-activator alpha (PGC-1α).[111, 112] Together, reduced mitochondrial biogenesis and overproduction of ROS contribute to further progression of heart failure disease. This also represents another potential target of future heart failure therapies.

## 2.6 Hypertension and heart failure

Hypertension is one of the most important risk factors for the development of heart failure.[113] Apart from heart failure, hypertension also increases the risk of stroke, coronary artery disease and renal failure.[114,115] Other risk factors for heart failure include diabetes, myocardial infarction and valve disease.[8] Hypertension is defined as systolic and diastolic pressures greater than 140 and 90 mm Hg, respectively.[115] The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure categorized hypertension into the following grades: pre-hypertension (120-139/80-89 mm Hg), stage 1 hypertension (140-159/90-99 mm Hg) and stage 2 hypertension: (>160/>100 mm Hg).[115] However, the most recent 2017 ACC/AHA hypertension guidelines recommend a lower blood pressure threshold of >130/>80 mm Hg as the cut-off for elevated blood pressure.[116]

Hypertension may also be classified as essential (primary) or non-essential (secondary) based on the nature of the underlying causes. Essential hypertension is idiopathic in nature and accounts for about 90-95% of all hypertension cases.[117] Secondary hypertension (5-10%) occurs due to identifiable underlying causes such as renovascular disease, primary renal disease, primary aldosteronism or use of medications (e.g. oral contraceptive, non-steroidal anti-inflammatory drugs, stimulants).[118]

Arterial blood pressure is a product of cardiac output and systemic vascular resistance. While the underlying cause of essential hypertension is unknown, factors that could influence cardiac output and vascular resistance include alteration of RAAS, overactivation of the sympathetic nervous system and plasma volume, regulated by the kidney.[119] Thus, essential hypertension mostly responds to antihypertensive medications that target the RAAS and sympathetic nervous system.

Conversely, secondary hypertension may be resistant to antihypertensive drugs and is best treated by addressing specific underlying causes.[119]

**From hypertension to heart failure**

Hypertension is a classical example of pressure overload conditions, since the heart is forced to pump against high aortic and arterial pressures. Prolonged hypertension exerts considerable pressure on ventricular walls and leads to left ventricular hypertrophy. Initially, thickening of the ventricular walls tends to offset high wall stress.[120] This stage is known as compensated hypertrophy. After a prolonged time, it becomes increasingly difficult for the hypertrophied ventricular walls to relax and fill adequately, resulting in diastolic dysfunction and decompensated hypertrophy.[121] Indeed, the prevalence of left ventricular hypertrophy is higher in hypertensive patients compared to the healthy population.[122] In addition, left ventricular hypertrophy increases the risk of adverse cardiovascular events.[123] The shift from compensated hypertrophy to overt heart failure is characterized by several changes in the myocardium such as fibrosis, apoptosis and altered coronary circulation.[124] Alternatively, hypertension may also increase the risk of myocardial infarction and ischemic heart disease leading to systolic dysfunction and heart failure.[125]

## 2.7 Drugs used in the treatment of HFrEF

The treatment plan for HFrEF should include both pharmacological and non-pharmacological strategies. Non-pharmacological strategies involve exercise training, salt and water restriction and weight management.[35] In some cases, device intervention such as implantable cardioverter defibrillator, left ventricular assist or cardiac resynchronization therapy may be required. Heart transplantation is mostly reserved for refractive NYA class IV cases.[35]

According to the Canadian Cardiology Society (CCS) algorithm for treatment of HFrEF, acute symptoms such as fluid retention should be relieved first by administering diuretics. An Angiotensin Converting Enzyme Inhibitor (ACEI) should also be initiated as soon as possible.[31] β-blockers may cause exacerbation of acute heart failure symptoms by worsening the patient's hemodynamic status and thus should only be started after initial symptoms have subsided.[126] In patients that do not tolerate ACEI due to cough, an angiotensin receptor blocker (ARB) can be used instead. In patients that continue to experience symptoms despite treatment with an ACEI and β-blocker, an ARB can be added.[31]

**Diuretics:** in addition to salt and water restriction, diuretics are used to relieve clinical symptoms of volume overload. There are different types of diuretics but the loop diuretic is the most commonly used in heart failure patients (e.g. furosemide, torsemide, bumetanide).[127] Loop diuretics achieve more efficient diuresis compared to thiazide diuretics that act in the distal convoluted tubule, where only 5 - 8% of filtered sodium is reabsorbed. Loop diuretics inhibit $Na^+$-$K^+$-$Cl^-$ co-transporters in the ascending loop of Henle, where 20 - 25% of filtered sodium is normally reabsorbed.[63, 127] When adequate diuresis cannot be achieved by high doses of furosemide, torsemide or bumetanide should be used due to greater, and less variable, bioavailability.[128-130]

**Digoxin:** improves cardiac contractility by inhibiting $Na^+$-$K^+$ ATPase leading to increased intracellular sodium and calcium concentrations.[131, 132] Digoxin has been used in the treatment of heart failure for centuries.[133] While there is evidence that digoxin improves symptoms in heart failure with systolic dysfunction, there is no evidence that it improves survival, at least according to the DIG trial.[134] Digoxin is not recommended for patient stabilization during acute exacerbation and should only be initiated after stabilization of acute symptoms in addition to other first line

drugs. The therapeutic window for digoxin is very narrow and thus therapeutic levels should be monitored closely to be within the recommended range (0.5-0.9 ng/mL).[135] Higher serum levels have been associated with toxicity and higher mortality rate.[136]

**β-blockers:** by blocking beta adrenergic receptors, β-blockers reduce the adverse effects of over-activation of the sympathetic nervous system that occur in heart failure. In addition, β-blockers reduce circulating levels of vasoconstrictors such as NE and ET-1, thus slowing the rate of progression of cardiac dysfunction. β-blockers should not be initiated in unstable and hospitalized patients.[126] Initiation of β-blockers at target doses could lead to acute exacerbation and worsening of heart failure symptoms. Thus they should be initiated at low doses and titrated up slowly until target doses are achieved and should not be stopped abruptly.[31] There is strong evidence from clinical trials that the use of β-blockers reduces both mortality and morbidity in patients with systolic heart failure.[135, 137, 138] β-blockers commonly used include carvedilol, bisoprolol and extended release metoprolol succinate. In a double-blinded, multi-center randomized control trial (CBIS-II), bisoprolol reduced all-cause mortality, sudden death and hospitalizations in NYHA class III and IV patients receiving standard therapy compared to placebo.[139] Although a high dose of metoprolol succinate (200 mg once daily) improved survival, improved symptoms and hospitalization in the MERIT-HF studies,[140, 141] carvedilol was superior to metoprolol tartrate (50 mg twice daily) in reducing mortality in a comparison trial of carvedilol and metoprolol (COMET).[142]

**ACEI:** ACEI inhibits the conversion of Ang I to Ang II and thus reduces the effects of over-activation of RAAS. The beneficial effects of ACEI surpass vasodilatory effects since hydralazine, an arterial vasodilator, does not regress ventricular hypertrophy.[143, 144] Several randomized trials have shown that ACEI significantly improve survival and symptoms in patients with heart failure

with systolic dysfunction.[145-149] In the CONSENSUS study, enalapril reduced HF progression and mortality, and improved symptoms compared to placebo in NYHA stage IV patients while on conventional heart failure therapy.[145] In another trial focussed on asymptomatic patients, with ejection fraction less than 35%, who were not receiving any treatment for heart failure, enalapril also reduced incidence and hospitalization for heart failure compared to placebo within 37 months follow-up period.[148] Thus ACEI have formed the cornerstone for heart failure therapy.

**ARBs** target the same hormonal system as ACEI albeit slightly differently. ARB competitively inhibits the binding of Ang II with angiotensin II receptor type-1 (AT-1 receptors) thereby reducing the maladaptive effects of Ang II.[33] Since ARBs do not affect breakdown of bradykinin, they do not cause dry cough, which is a common side effect of ACEI. Similar to ACEI, ARBs also reduce heart failure symptoms and hospitalizations but barely reduce mortality in heart failure patients when compared to placebo.[150] ARBs are used as second line when ACEI are not tolerated by the patients or as an adjunct to ACEI. However, routine combination of an ACEI and ARB is not generally recommended.[31, 135] The use of valsartan, candesartan, and losartan has been studied in clinical trials,[151-153] and candesartan was associated with lower mortality rates compared to losartan.[154]

**Aldosterone antagonists** directly inhibit binding of aldosterone to aldosterone receptors and prevent sodium-potassium exchange in the distal renal tubule. This leads to sodium and water excretion and potassium retention. Drugs in this class include spironolactone and eplerenone. The RALES study showed that spironolactone reduced mortality and symptoms in NYHA class III and IV patients (treated with ACEI, loop diuretics and digoxin) compared to placebo.[155] Moreover in the EMPHASIS trial, eplerenone reduced mortality and improved symptoms in NYHA class II heart failure patients versus placebo, in addition to recommended therapy.[156] Therefore, these

drugs are recommended for use in patients with persistent symptoms despite use of ACEI, ARB and β-blockers.[31]

**Angiotensin receptor-neprilysin inhibitor (ARNI):** Sacubitril/valsartan (Entresto) was approved in 2015 by the FDA and added to the ACC/AHA heart failure treatment guidelines.[157] Entresto is a combination of a neprilysin inhibitor (sacubitril) and an ARB (valsartan) and is approved for the treatment of heart failure with reduced ejection fraction (HFrEF) in the US and Canada.[157, 158] Neprilysin is a membrane-bound peptidase that breaks down endogenous vasoactive peptides such as natriuretic peptides (ANP and BNP), bradykinin and adrenomedulin.[159-161] Inhibition of neprilysin thus increases levels of these natriuretic peptides and improves hemodynamic status in heart failure by counter-acting the deleterious effects of RAAS overactivation.[162, 163] In the Prospective Comparison of Angiotensin Receptor-Neprilysin Inhibitor [ARNI] with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) trial, Entresto was superior to enalapril in reducing risks of mortality and hospitalization due to heart failure in addition to recommended therapy.[164] Side effects include hypotension, renal insufficiency and angioedema.[157]

**Funny ($I_f$) Current inhibitor:** Ivabradine (Corlanor) is another newly approved drug which is recommended for use in stable HFrEF where heart rate is greater than 70 beats per min despite β-blocker therapy. It acts by inhibiting the $I_f$ current in the SA node, thus regulating heart rate. In a randomised placebo-controlled trial conducted in heart failure patients with heart rates greater than 70, who have been on conventional therapy including β-blockers, ivabradine reduced the risk of hospitalization and cardiovascular death by 18%.[165] Greater benefits were observed for those with higher heart rates. Ivabradine however did not exhibit clinical benefit in patients with stable coronary artery disease with or without reduced ejection fraction.[166, 167]

## 2.8 Drugs used in the treatment of HFpEF

Conventional drugs used in HFrEF patients do not improve morbidity and mortality in HFpEF patients. For example, in the DIG trial, digoxin (in addition to diuretics and ACEI) did not improve symptoms nor prevent deaths among HFpEF patients.[168] Furthermore, the CHARM I-preserve trial tested the effects of candesartan versus placebo in HFpEF (ejection fraction >45%) in addition to conventional treatments. There was no improvement in primary outcomes such as death and hospitalizations due to heart failure.[169] Treatment for HFpEF is therefore limited to ameliorating symptoms. Blood pressure is adequately controlled with antihypertensive agents, preferably ACEI or ARB. Fluid retention is managed with diuretics, heart rate is controlled by β-blockers, and diabetes is treated by appropriate anti-hyperglycemic agents.[135]

In the 2015 update to Canadian guidelines, spironolactone is recommended for the treatment of HFpEF[158] based on results of the TOPCAT trial. Here, although spironolactone did not improve outcomes in the general analysis, a post-hoc analysis revealed benefits in a subgroup of HFpEF patients with high BNP levels.[170] Close monitoring of serum potassium and renal function is recommended when spironolactone is used.[158]

## 2.9 Cardiac hypertrophy

Cardiac hypertrophy is an increase in myocardial mass induced by hemodynamic stress or injury to the heart.[171] Although cardiac hypertrophy was thought to be a compensatory mechanism to stress or injury,[10] it is now established that prolonged hypertrophy leads to functional decompensation.[11-13] In epidemiological human studies, prevention and regression of cardiac hypertrophy has been associated with better prognosis in cardiovascular diseases. In the Heart Outcomes Prevention Evaluation (HOPE) trial, administration of ramipril to high risk patients reduced left ventricular hypertrophy[172] and risk for adverse cardiovascular outcomes[19] compared to placebo. Furthermore, in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE trial), losartan was superior to atenolol in reducing left ventricular hypertrophy and prevention of cardiovascular morbidity and mortality.[20] Thus prevention of cardiac hypertrophy proves to be a potential strategy for the mitigation of adverse cardiovascular outcomes.[14]

Cardiac hypertrophy can be classified as concentric or eccentric hypertrophy which may be due to pressure or volume overload, respectively.[16] Examples of pressure overload conditions include hypertension and aortic stenosis. Consistently high end-systolic pressures cause addition of sarcomeres in parallel which leads to concentric hypertrophy, characterized by increased ventricular wall thickness with little or no change in the chamber volume.[17] Conversely, volume overload may result from mitral valve regurgitation, in which case blood pools in the heart.[18] Eccentric hypertrophy involves an increase in chamber volume without increase in wall thickness. The heart muscles are elongated by addition of sarcomeres in series. In addition, prolonged hypertrophy causes dilated hypertrophy, which is the decompensated state of the heart. In decompensated hypertrophy, characterized by myocyte degeneration and replacement fibrosis, the ventricular chamber becomes excessively dilated and the walls excessively thin.[173, 174]

Cardiac hypertrophy can also be classified as physiologic or pathologic. Physiologic hypertrophy occurs when the myocardial muscle mass increases due to increased energy demand such as during pregnancy,[175] exercise,[176, 177] and post-natal growth.[178, 179] In physiologic hypertrophy, the increase in myocardial mass is accompanied by a corresponding increase in capillary network, and cardiac structure and function are maintained.[180, 181] In contrast, pathologic hypertrophy is induced by hemodynamic stress or injury to the heart such as hypertension, myocardial infarction and arrhythmia. Lack of commensurate growth in capillary network along with the increase in myocyte size leads to hypoxia, higher energy demand and thus abnormal function.[182] The difference between physiologic and pathologic hypertrophy lies first, in the stimuli that initiate the process and second, the maladaptive processes activated during pathologic hypertrophy.

Physiologic hypertrophy is fully reversible upon removal of the hypertrophic stimuli. For instance, left ventricular hypertrophy associated with pregnancy is usually resolved at about 8 weeks post-partum[183] while detraining athletes also reverses physiologic hypertrophy within a period of 6-34 weeks.[184] Conversely, complete regression of pathologic hypertrophy is more difficult to achieve. Regression of pathologic hypertrophy may be achieved by addressing the underlying etiology.[185] For instance the use of antihypertensive[186] and aortic valve replacement[187] regressed left ventricular hypertrophy. However, in decompensated hypertrophy, the underlying pathology such as cardiomyocyte death and myocardial fibrosis may not be completely reversible.[188] In addition, sometimes regression of hypertrophy does not translate into improved function and electrophysiology. Thus, prevention rather than regression of hypertrophy seems to be a better therapeutic strategy.

In contrast to physiologic hypertrophy, pathologic hypertrophy is associated with activation of the fetal gene program (i.e. ANP, BNP and α-skeletal actin),[189] cardiac fibrosis,[190] cell death by

apoptosis and necrosis,[174] sarcomere disorganization and capillary rarefaction.[182] Furthermore, there is down-regulation of calcium-handling proteins such as SERCA[191] and reduced expression of contractile genes such as myosin heavy chain.[192] Other types of pathologic hypertrophy include genetic hypertrophic cardiomyopathy[193] and diabetic cardiomyopathy, which is characterized by myocardial functional and structural abnormalities independent of coronary heart disease or hypertension.[194, 195] Distinct signaling mechanisms are involved in physiologic and pathologic hypertrophy.

### 2.9.1 Physiologic hypertrophic signaling

The best characterized signaling pathway involved in physiologic hypertrophy is insulin or insulin-like growth factor signaling-1 (IGF-1).[196] Other growth hormones involved in physiologic hypertrophy include thyroid hormone and vascular endothelial growth factor.[196] IGF-1 is produced primarily in the liver in response to growth hormone release from the pituitary gland.[197] IGF-1 mediates cell growth in various tissues including the heart,[198] and acts by binding to tyrosine kinase insulin receptors and activating a lipid kinase, PI3K (phosphoinositide-3-kinase). PI3K phosphorylates a membrane lipid, PIP2 (phosphatidylinositol-4,5-diphosphate), and activates PIP2-dependent protein kinase (PDK). PDK further activates AKT-1 (protein kinase B) downstream. AKT-1 is associated with increased protein synthesis via activation of mammalian target of rapamycin (mTOR),[199] increased glucose uptake, and glycogen synthesis leading to increase in cell size.[200]

Cardiac levels of IGF-1, but not ET-1 or Ang II, are higher in professional soccer players compared to non-athletic controls.[201] Serum levels of IGF-1 were also increased after intense exercise training in humans[202] and in rats.[203] In a study by McMullen *et al.*, cardiac-specific overexpression of the IGF-1 gene in mice resulted in cardiac hypertrophy with enhanced systolic function and no

signs of histopathology.[204] This supports the role of IGF-1 in physiologic hypertrophy. Moreover, the downstream targets of IGF-1 such as PI3K and AKT levels were elevated in the transgenic hearts while signaling molecules known to be involved in pathologic hypertrophy such ANP and BNP remained inactivated.[204] In two contrasting transgenic mouse models, overexpression of PI3K resulted in mice with larger hearts and activation of Akt, while mice with reduced activity of PI3K exhibited smaller hearts and reduced Akt levels. Notably, cardiac function and life span were normal in both cases and markers of pathologic hypertrophy (ANP and BNP) were unaffected.[205] In another study by McMullen *et al.*, PI3K was essential for the induction of physiologic but not pathological cardiac growth. In this study, dominant-negative PI3K mutant transgenic mice developed cardiac hypertrophy and systolic dysfunction when exposed to pressure overload but not to exercise training.[206] This suggests that PI3K plays a role in determining heart size in physiologic hypertrophy.

### 2.9.2 Pathologic hypertrophic signaling

Pathologic hypertrophy occurs in response to sustained hypertension, myocardial infarction and activation of neuro-hormonal mechanisms. Activation of neuro-hormonal mechanisms leads to higher circulating levels of vasoactive substances such as Ang II, endothelin-1 (ET-1) and catecholamines. Indeed, high levels of circulating Ang II,[207] NE[208] and ET-1[209] occur in heart failure patients. In animal and experimental models, cardiac hypertrophy can be induced by hypertrophic stimuli such as ET-1, Ang II or epinephrine. These agents bind to a family of receptors known as G-protein coupled receptors (GPCR) on cardiomyocytes to induce hypertrophy. ET-1 binds to ET-1 receptors, Ang II to Ang II (AT) receptors and epinephrine to α and β-adrenergic receptors.

GPCRs are a group of transmembrane receptors which have ligand-binding sites on the outside of the cells and are coupled to G-proteins on the inside. G-proteins refer to guanine nucleotide-binding proteins that bind to GDP or GTP in their inactive or active states respectively. Binding of the ligand to a GPCR leads to conformational changes in the receptor and activation of the G-protein. G-protein consists of 3 subunits: α, β and γ. After activation, the α-subunit of the G protein dissociates from the β-γ complex and is released to activate downstream signaling molecules in the cells to achieve intracellular response. The 3 types of G-proteins are $G_{\alpha s}$, $G_{\alpha q/\alpha II}$ and $G_{\alpha i}$ and they play different roles which include modulating contractility and hypertrophy in the heart. $G_{\alpha s}$ is activated by binding of epinephrine to β-adrenergic receptors and effects activation of adenylyl cyclase. $G_{\alpha i}$ is activated by binding of inhibitory ligands such as adenosine to adenosine receptors resulting in inhibition of adenylyl cyclase. Adenylyl cyclase catalyses the conversion of adenosine monophosphate (AMP) to cyclic AMP, which activates protein kinase A and regulates contractility and other physiological processes.

There are many downstream signaling molecules implicated in the development of pathologic cardiac hypertrophy. $G_{\alpha q/\alpha II}$ is activated by binding of epinephrine, Ang II and ET-1 to $\alpha_1$-adrenergic receptors, AT-1 receptors and ET-1 receptors respectively. This leads to activation of phospholipase C, which then breaks down the membrane lipid PIP2 into IP3 (inositol-1,4,5-triphosphate) and DAG (diacylglycerol).[210] IP3 and DAG serve as second messengers to activate other signaling molecules further downstream such as PKC (protein kinase C) and CAMK II (calcium calmodulin-dependent protein kinase II) as reviewed by van Berlo *et al.*[211]

Other signaling pathways involved in pathologic hypertrophy include MAPK (mitogen activated protein kinase) and calcineurin-nuclear factor of activated T-cells (NFAT) and have been extensively reviewed recently by Shimizu *et al.*[212] The focus of my research is on the role of

AMPK (AMP-activated protein kinase) and MITF (microphthalmia-associated transcription factor) signaling molecules in pathologic cardiac hypertrophy.

## 2.10 AMP-activated protein kinase (AMPK)

AMPK is a heterotrimeric enzyme that acts as an intracellular sensor of energy balance. AMPK regulates cell growth, differentiation and metabolism under low energy conditions by phosphorylating key metabolic enzymes and transcription factors.[213] It is a serine-threonine kinase consisting of one catalytic (α) subunit and two regulatory subunits (β and ϒ). AMPK was originally purified from rat liver.[214] Each subunit exists as distinct isoform types. For instance, there are two isoforms of the AMPK α-subunit: AMPKα1 and AMPKα2.[215, 216] AMPKα isoforms are expressed in mammalian heart, skeletal muscles, liver, kidney, lung and secretory cells.[216, 217] In humans, expression of the AMPKα2 isoform is more predominant in the heart while AMPK α1 is expressed more in secretory cells.[217] In rat heart, activity of AMPKα2 was two- to three-fold greater than AMPKα1 under normal and ischemic conditions.[218]

During energy-deprived conditions, a high AMP-ATP ratio drives activation of AMPK via phosphorylation at Thr-172 by upstream AMPK kinases such as tumor suppressor kinase LKB1[219, 220] and calcium-calmodulin dependent protein kinase kinase (CAMKKβ).[221] Although not involved in AMPK activation, other sites of phosphorylation include the Thr-258 and Ser-485/491 positions.[222] The exact role of these additional AMPK phosphorylation sites is not fully understood. Phosphorylation of AMPK at Ser-485/491 may induce inhibition of AMPK activity.[223] The action of AMPK is inhibited via dephosphorylation by protein phosphatase 2A, in response to intracellular calcium changes,[224] and protein phosphatase 2C.[225] After activation, AMPK acts by phosphorylating downstream targets, leading to a reduction in ATP-consuming pathways and an increase in ATP-generating pathways.[226] There are many pharmacological agents that directly or

indirectly activate AMPK.[227] Metformin and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) are mostly used as experimental AMPK activators.[228, 229] Dorsomorphin (compound C) is a small molecule, cell-permeable AMPK inhibitor that is also used as an experimental tool to understand the effects of AMPK.

### 2.10.1 AMPK and metabolism

During periods of metabolic stress, AMPK conserves energy by repressing energy-consuming pathways (such as protein synthesis) and activating energy-generating processes. Activation of AMPK modulates major signaling molecules involved in protein synthesis such as eukaryotic elongation factor 2 (eEF2),[230] mammalian target of rapamycin (mTOR),[231] P70-S6 kinase P70S6K[232] and tuberous sclerosis 2 (TSC-2),[233] resulting in decreased protein synthesis. eEF2 is required for peptide chain elongation, an essential step in protein synthesis. AMPK inhibits protein synthesis via activation of eEF2 kinase, which then phosphorylates and inactivates eEF2.[230] mTOR is a serine threonine kinase which promotes protein synthesis by phosphorylating key proteins required for the initiation of protein translation such as P70 S6 kinase. P70 S6 kinase phosphorylates the ribosomal protein P70 S6 and induces protein synthesis at the ribosome. Activation of AMPK inhibits the actions of mTOR and as such inhibits protein synthesis. TSC-2 regulates protein synthesis by inhibiting the actions of mTOR. TSC-1 and TSC-2 interact to form a complex, which acts as a tumor suppressor, inhibiting the activity of mTOR. AMPK activates the TSC1-TSC2 complex via phosphorylation of 2 residues on TSC2.

Energy-generating processes activated by AMPK include the following: AMPK activation induces mitochondrial biogenesis via upregulation of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1α) [234, 235] and activation of sirtuin-1.[236, 237] AMPK activation also increases fatty acid uptake and fatty acid utilization to generate ATP.[238] Furthermore, AMPK increases fatty

acid oxidation by phosphorylating and inactivating downstream acetyl-CoA carboxylase (ACC) and increasing activity of carnitine palmitoyl transferase (CPT)-1.[239] CPT-1 aids fatty acid transport into the mitochondria, where fatty acids are oxidized to generate ATP. AMPK activation by AICAR increases glucose uptake by increasing translocation of glucose transporter type 4 (GLUT4) to the sarcolemma.[240] Moreover, AMPK increases glycolysis via phosphorylation and activation of 6-phosphofructo-2-kinase (PFK-2), a potent stimulator of glycolysis in ischemic rat hearts[241]

### 2.10.2 AMPK and cardiac hypertrophy

In the healthy heart, AMPK phosphorylation and activity levels increase during exercise in response to high AMP levels and energy demands.[242] Studies by Musi *et al*. showed that AMPKα2 activity in mouse heart increased more dramatically than AMPKα1 in response to treadmill exercise.[243] AMPK is also activated in disease conditions such as ischemia.[238, 244]

Pharmacological activation of AMPK by metformin and AICAR inhibited phenylephrine-induced protein synthesis in neonatal rat cardiomyocytes.[232] In this study, metformin and AICAR increased phosphorylation of AMPK at Thr-172 and ACC (a direct substrate of AMPK) at Ser-79 in the presence and absence of phenylephrine. Phenylephrine and Akt overexpression induced cardiomyocyte hypertrophy (increased myocyte size and protein synthesis) and decreased phosphorylation of eEF2. This hypertrophic response was inhibited by metformin and AICAR. Thus, the authors concluded that metformin and AICAR inhibited protein synthesis via downregulation of eEF kinase-eEF signaling and/or the P70 S6 kinase pathway.[232] Activation of AMPK by AICAR inhibited cardiomyocyte growth and β-MHC gene expression induced by phenylephrine partly via modulation of atrophy-related FOXO/MuRF1 signaling.[245]

Furthermore, AMPK activation inhibited Ang II-induced cardiomyocyte hypertrophy by preventing associated metabolic changes.[246] In this study, AICAR increased AMPK Thr-172 phosphorylation, decreased glucose uptake, inhibited protein synthesis while increasing fatty acid utilization.[246] Resveratrol, a polyphenol found in grapes and red wine, inhibited neonatal rat cardiomyocyte hypertrophy (induced by phenylephrine) via activation of AMPK and suppressed protein synthesis by inhibiting Akt.[24]

Infection of cardiomyocytes with an AMPK-expressing adenovirus inhibited cardiomyocyte size increase, protein synthesis as well as ANP and BNP fetal gene induction in Ang II-induced cardiomyocyte hypertrophy.[247] Moreover, subcutaneous administration of AICAR for 7 weeks inhibited cardiac hypertrophy in a pressure-overload model (trans-aortic constriction [TAC]) of cardiac hypertrophy.[247] Here, phosphorylated AMPK levels were higher in the TAC group compared to sham control, and AICAR drove phosphorylated AMPK levels even higher. Notably, activity of AMPKα2, but not AMPKα1, was increased in the TAC group and was further increased with administration of AICAR.[247]

Activation levels of AMPK in the heart during cardiac hypertrophy vary depending on the model. Phosphorylated AMPK protein levels were higher in pressure overload achieved by aortic constriction.[17,248] In contrast, Thandapilly *et al.*[26] and Dolinsky *et al.*[249] reported lower levels of AMPK phosphorylation in the spontaneously hypertensive rat (SHR) at 20 weeks and 15 weeks respectively. Activation of AMPK by phosphorylation was also reduced in NE-induced hypertrophy (24-hour treatment) in adult rat cardiomyocytes[26] and Ang II-induced hypertrophy (4-hour treatment) in neonatal rat cardiomyocytes[246] but was unchanged in phenylephrine-induced hypertrophy (24-hour treatment) in neonatal rat cardiomyocytes.[24]

Despite evidence showing protective effects of AMPK activation on cardiac hypertrophy, other authors have shown that cardiac AMPK activation may instead be correlated to cardiac hypertrophy.[248, 250] In a study by Tian *et al.*, activities of AMPKα1 and AMPKα2 isoforms were significantly elevated in a pressure-overload (ascending aortic constriction) model of cardiac hypertrophy.[248] However, disparate protein expression levels were observed between AMPK isoforms. AMPKα1 protein level was increased while AMPKα2 was reduced.[248] Elevated activity of the AMPKα2 isoform was not associated with an increase in AMPKα2 protein levels.

In another study, elevated AMPK activity was reported in a transgenic mice model overexpressing PRKAG2, the ϒ2 subunit of AMPK.[251] This transgenic mice model also exhibited left ventricular hypertrophy and accumulated large amounts of glycogen.[251] Glycogen accumulation itself is known to cause cardiac hypertrophy.[252] Therefore, it is unclear whether hypertrophy observed in this model was due to increased AMPK activity or glycogen accumulation.

Activation of AMPK during periods of stress or energy-deprived conditions may occur as a compensatory response to reinstate homeostasis. Perhaps increased AMPK activity associated with hypertrophy observed in the studies above is an example of such response. Innate activation of AMPK in these conditions without any other intervention may be insufficient to inhibit hypertrophy. Therefore, administration of drugs that also activate AMPK at the onset of hypertrophic stimuli may augment the compensatory mechanism and enhance the inhibitory effects on cardiac hypertrophy and other maladaptive processes.

## 2.11 Microphthalmia associated transcription factor (MITF)

MITF is a helix-loop-helix leucine zipper transcription factor, known to be expressed in melanocytes,[253] mast cells,[254] and osteoclasts[255] where it regulates gene expression. A specific isoform (MITF-H) has also been detected in high amounts in cardiomyocytes.[256] MITF isoforms

are expressed in various tissues. For instance, MITF-M is specifically expressed in melanocytes while several isoforms such as MITF-MC, C, H, A and M are expressed in mast cells.[256] In humans, mutation of the MITF gene causes Wardenburg syndrome II, a rare genetic condition characterized by deafness and hypopigmentation.[257, 258]

MITF is activated by phosphorylation at Ser 73[259] and Ser 409,[260] via upstream phospho-ERK and ribosomal s6 kinase pathways in melanocytes. Another phosphorylation site found in the Wardenburg syndrome II is the Ser-298 residue.[261] Phosphorylation of MITF leads to co-activation of other transcription factors and transcriptional activities, ubiquitination and finally proteasome-mediated degradation.[262] Thus activated MITF, per se, is short-lived.[263]

MITF is the best characterized member of the MiT family of transcription factors. The role of MITF in melanogenesis has been widely studied and reported. MITF binds to E-box elements in the promoter region of target genes.[264] For example, in melanocytes and melanoma, MITF regulates target genes involved in cell proliferation (TBX2 and CDK2), cell survival (BCL2) and differentiation.[262] MITF increases transcription and expression of tyrosinase enzyme, TYRP (tyrosinase-related protein) 1 and 2, key enzymes required in melanogenesis.[265]

### 2.11.1 MITF and cardiac hypertrophy

The role of MITF in cardiac function is not yet fully understood. While MITF was reported to be decreased in failing heart samples,[266] Tshori *et al*. showed specific involvement of MITF in the development of cardiac hypertrophy using two different MITF transgenic mouse models.[267] In one transgenic model, a stop codon was inserted in the MITF gene between the helix-loop-helix (HLH) and the ZIP domain. This resulted in expression of truncated MITF that was unable to bind appropriately. In the second model, about 50 copies of transgenes were incorporated in the MITF promoter, making the mice unable to express MITF. Here, both MITF-mutated mouse strains

showed reduced hypertrophic response to β-adrenergic stimulation (i.e. failure of fetal gene program induction), decreased cardiac function and tendency for sudden death.[267] Although there was no significant hypertrophic response to isoproterenol in mice carrying MITF mutations, some of the MITF-mutated mice suffered sudden death immediately after isoproterenol infusion.[267] Therefore, authors suggested arrhythmia as a cause for the sudden death. Authors also proposed that while MITF is required for the hypertrophic response, it is also required for normal heart function. Similarly, transduction of H9C2 cells by lentiviral vector expressing siRNA targeted at MITF resulted in 40% reduction in BNP promoter activity. This suggests that MITF, at least in part, regulates BNP promoter activity.[267] Another study has established a similar role for MITF in hypertrophic responses as MITF knockdown reduced hypertrophic response induced by Ang II.[268]

Downstream targets of MITF-H that have been identified in cardiomyocytes include ErbB2-interacting protein (Erbin),[269] micro-RNA 541 (miR-541),[268] myosin light chain-1a (MLC-1a)[256] and GATA4.[270] MicroRNA's are non-coding RNAs that act by interfering with mRNA translation and/or degradation. miR-541 is an anti-hypertrophic microRNA. miR-541 inhibited Ang II-induced hypertrophy *in vitro*, where cardiac-specific overexpression of miR-541 in mice produced a blunted hypertrophic response to Ang II treatment.[268] MITF regulates cardiac hypertrophy by interacting with miR-541 and repressing its transcription.[268] Furthermore, mice carrying the same mutated MITF genes (described above), exhibited reduced MLC-1a expression compared to their wild type littermates.[256] Thus the expression of MLC-1a is thought to be regulated by MITF-H.[256]

Another target of MITF is Erbin, a negative regulator of hypertrophy.[271] Erbin is a member of the leucine-rich repeat and PDZ domain family of proteins. It consists of 17 leucine-rich repeats and one PDZ domain, the domain that binds with other proteins such as ErbB2. Erbin expression is reduced in murine cardiac hypertrophy and human end-stage heart failure.[271] An exaggerated

hypertrophic response was observed in transgenic mice that lack erbin expression compared to wild type mice, suggesting an inhibitory role for erbin in cardiac hypertrophy.[271] MITF increased expression of Erbin under basal conditions but repressed Erbin during isoproterenol-induced hypertrophy.[269]

Finally, MITF binds with the E-box element of the GATA4 promoter to activate expression of the pro-hypertrophic transcription factor, GATA4, in response to β-adrenergic stimulation.[270] Levels of MITF protein were increased in Ang II- and isoproterenol-induced hypertrophy (6 h treatment) in adult mouse cardiomyocytes. Similarly, MITF mRNA and protein levels were increased in TAC-induced cardiac hypertrophy compared to sham-operated animals, two weeks post-surgery.[270] Together, these data show that MITF plays some role in hypertrophic responses, and a few specific downstream targets have been identified to support this hypothesis. However, more research is required to fully elucidate the roles of MITF in cardiac hypertrophy.

### 2.11.2 AMPK and MITF cross talk

The MAPK pathway is one of the signaling pathways activated during hypertrophic response.[272] MITF may be activated by the upstream ERK pathway,[259] while AMPK activation inhibits ERK.[273] Thus, I speculated that activation of AMPK may modulate the transcription and/or activity of MITF, which is believed to be required for cardiac hypertrophy. A few studies have examined AMPK-MITF cross-signaling in melanocytes and melanoma cells.

In a study by Borgdorff *et al*., inhibition of AMPK by compound C downregulated MITF protein levels in melanoma cells.[274] Although MITF was initially activated (phosphorylated) at an early time point (4 h), phosphorylated MITF levels returned to basal levels after 24 h.[274] Thus, authors proposed that AMPK may have a dual effect on MITF whereby it inhibits MITF transcription via

repression of CREB-specific co-activator while also maintaining MITF protein expression via dephosphorylation of ERK upstream.[274]

In contrast, another study showed that metformin inhibited melanogenesis gene expression and inhibited MITF promoter activity in an AMPK-independent mechanism.[275] Here, metformin inhibited melanin production and MITF expression in cells carrying dominant-negative AMPK constructs, indicating that AMPK is not required for inhibition of melanogenesis by metformin.[275] While these studies were done in melanocytes, the effect of AMPK activation on MITF expression in cardiomyocytes and cardiac hypertrophy is unknown. We know that AMPK activation is implicated in the anti-hypertrophic effects of metformin.[229, 232] It is possible that AMPK activation also represses MITF expression during cardiac hypertrophy.

## 2.12 Stilbenoids

### 2.12.1 Stilbenoid structure

Stilbenoids are a group of naturally occurring non-flavonoid polyphenols found in various plant species.[276] They share a common backbone stilbene structure but differ in the type and position of substituents on the ring (Figure 3). Stilbenoids exist as monomers or oligomers. They may also be found free (aglycone) or conjugated as glucosides. For example, piceid is resveratrol-3-O-glucoside.[277, 278] Monomeric stilbene (trans-1,2-diphenylethylene) aglycone structure consists of two phenyl rings joined by an ethylene bridge. Stilbenes may exist as the cis- or trans-isomer, but the trans-isomer is the more common and stable configuration.[276]

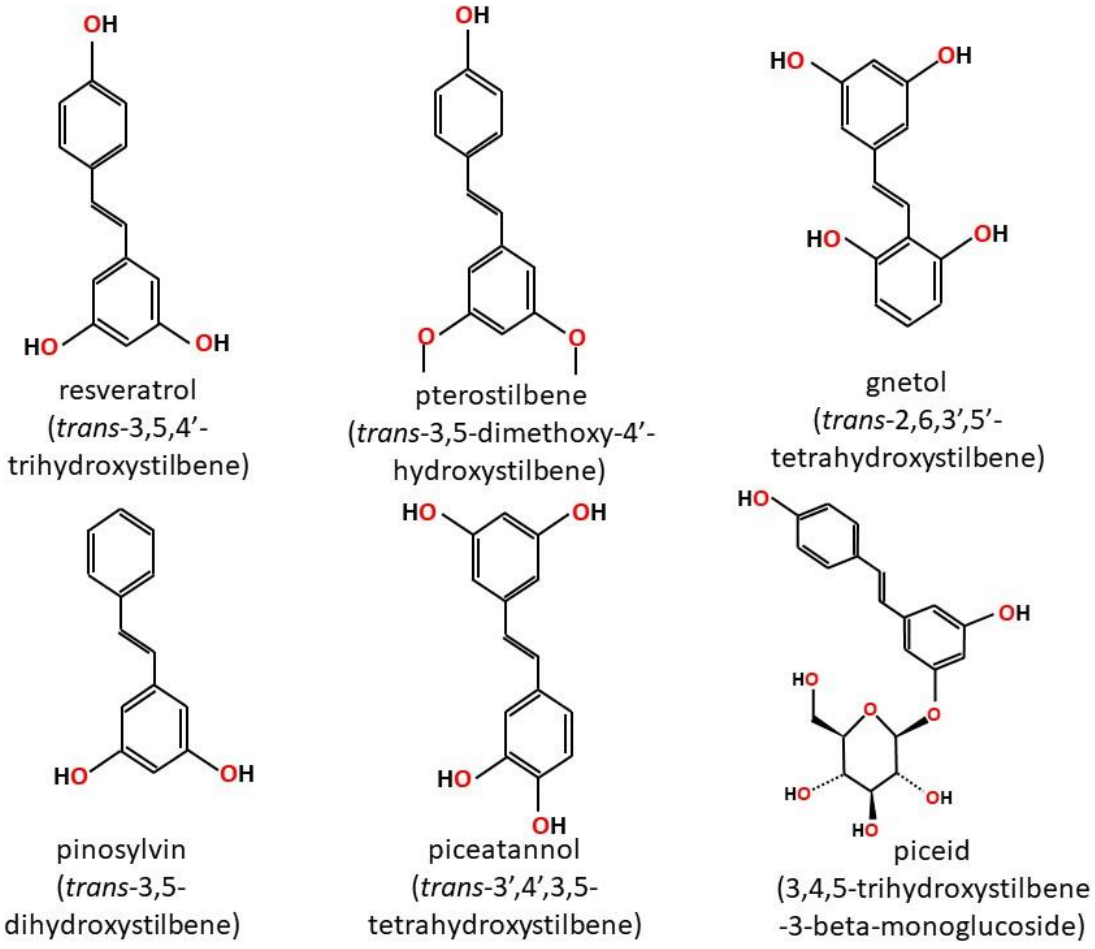



**Figure 3** Chemical structures of selected stilbenoids showing common stilbene backbone

The most popular and perhaps the most widely studied stilbenoids is resveratrol. Apart from resveratrol, there are other structural analogs with potentially beneficial medicinal properties. However, there is limited information about the biological effects of other stilbenoids.

### 2.12.2 Stilbenoid sources

Stilbenoids are naturally occurring phytoalexins, which are antimicrobial compounds produced *de novo* to protect the plant from fungal infection and toxins.[279, 280] Resveratrol (trans-3,5,4'-trihydroxystilbene) is found in *Vitis species* (grapes), red wine and other plant species.[279, 281] Pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene) is a dimethylether analog of resveratrol and is found in plant species such as *Pterocarpus marsupium*,[282] grapes,[280] and *Vaccinium species* (blueberries).[283] HPLC analysis revealed that resveratrol and pterostilbene are present in drakshasava, an ancient cardiotonic preparation used in Ayurvedic medicine.[284]

Gnetol (trans-2,6,3',5'-tetrahydroxystilbene) is another stilbenoid found in several species of the genus *Gnetum*. *Gnetum* is a gymnospermous plant with more than 35 species and occurs as trees, shrubs and lianas. Specifically, gnetol has been isolated from *Gnetum ula*,[285] *Gnetum gnemon*,[286] *Gnetum montanum*,[287] *Gnetum klossii*[288] and *Gnetum hainanese*.[289] The seeds and leaves of *Gnetum gnemon*, also known as melinjo, are eaten as vegetables in Indonesia.[290] Gnetol is used in folk medicine for arthritis and asthma.[30]

### 2.12.3 Bioavailability

Resveratrol has poor water solubility (< 0.05 mg/mL) and low oral bioavailability.[28] Complexation with cyclodextrins improves aqueous solubility of resveratrol but not bioavailability.[28] In this study administration of higher doses of resveratrol also did not improve the pharmacokinetic profile.[28] Other attempts to improve bioavailability of stilbenoids include complexation with bile acids,[291] incorporation into liposomes[292] and formulation into nanoparticle delivery systems.[293, 294]

The bioavailabilities and half-lives of selected stilbenoids following oral administration in rats are shown in Table 1. Pterostilbene exhibited the highest bioavailability of 80%[29] while gnetol exhibited the lowest oral bioavailability of 6.59%.[295] After intravenous administration, resveratrol exhibited a very short half-life of 14 min due to rapid metabolism. The reported bioavailability values for resveratrol range from 20 to 29.8%.[29, 296] Although gnetol has the lowest bioavailability, the reported half-life after oral administration of 100 mg/kg in rat was 4.2 h.[295] This value is significantly longer than values reported for resveratrol (1.48 h)[242] and pterostilbene (1.73 h).[297] Moreover, higher levels of gnetol glucuronide metabolite persisted for up to 72 h in serum after oral administration.[295] It is possible that gnetol glucuronide may be reconverted into free gnetol and thus compensate for the low bioavailability.

**Table 1: Half-lives and oral bioavailabilities of stilbenoids after oral administration in rats**

| Stilbenoid | Oral dose | Half life | Oral bioavailability (rats) |
|---|---|---|---|
| **Resveratrol** | 50 mg/kg | 1.48 h[296] | 29.8%[296] |
| **Pterostilbene** | 20 mg/kg | 1.73 h[297] | 80%[29] |
| **Gnetol** | 100 mg/kg | 4.2 h[295] | 6.59%[295] |

The presence of two methoxy groups in the pterostilbene structure makes it more lipophilic and thus more bioavailable.[29] Pterostilbene is also more metabolically stable because it has only one free hydroxyl group. Thus, based on the pharmacokinetic profile of stilbenoids, pterostilbene is more bioavailable than resveratrol and as such may be a potential candidate as an alternative to resveratrol.

### 2.12.4 Metabolism

Resveratrol undergoes glucuronidation in the intestine and is mainly absorbed as the glucuronide.[298, 299] The remaining resveratrol, absorbed as the aglycone, is further metabolized in the liver to sulphates and glucuronides.[300] The low bioavailability of stilbenoids is largely due to rapid and extensive metabolism in the intestine and liver during and after absorption giving rise to a lower levels of the free parent compounds.[301] Stilbenoids are biotransformed into sulphates and glucuronides through the actions of sulphotransferases (SULT) and UDP-glucuronosyltransferases (UGT), respectively. Resveratrol has been shown to be a better substrate for glucuronidation than pterostilbene.[302] The common metabolites of resveratrol and pterostilbene are shown in Figure 4 and Figure 5 below. The main metabolite of gnetol was found to be the glucuronide but the specific positions of predominant glucuronidation is yet to be identified.[295]

Despite the relatively low bioavailability of parent resveratrol, many studies have shown its biological activities *in vivo* in various animal studies. Pharmacokinetic studies showed high levels of stilbene metabolites (sulphates and glucuronides).[303] Therefore some argue that these metabolites may act as reservoirs for the stilbenoids either by direct action of metabolites[304] or via enterohepatic recycling.[296, 303] The biological activities of stilbene metabolites vary widely. While some studies have reported little or no effects of some metabolites,[305] others reported comparable or greater activities compared to the parent compounds.[304, 306] Furthermore, the presence of sulphatases and glucuronidases in some tissues support the possibility of reconversion of metabolites back to their parent compounds and may account for the activities of stilbenoids *in vivo*.[305, 307] Thus, biological effects may still be attained with low circulating levels of the parent stilbenoid compounds.

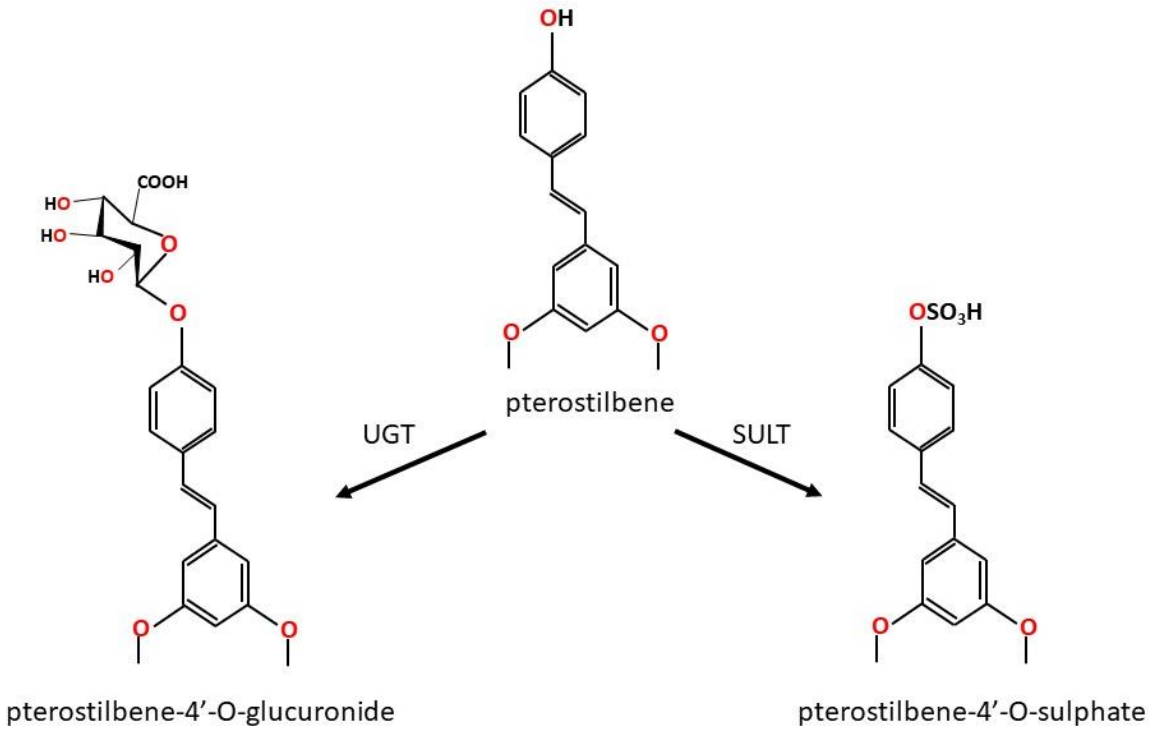



**Figure 4: Metabolic pathways and common metabolites of pterostilbene**

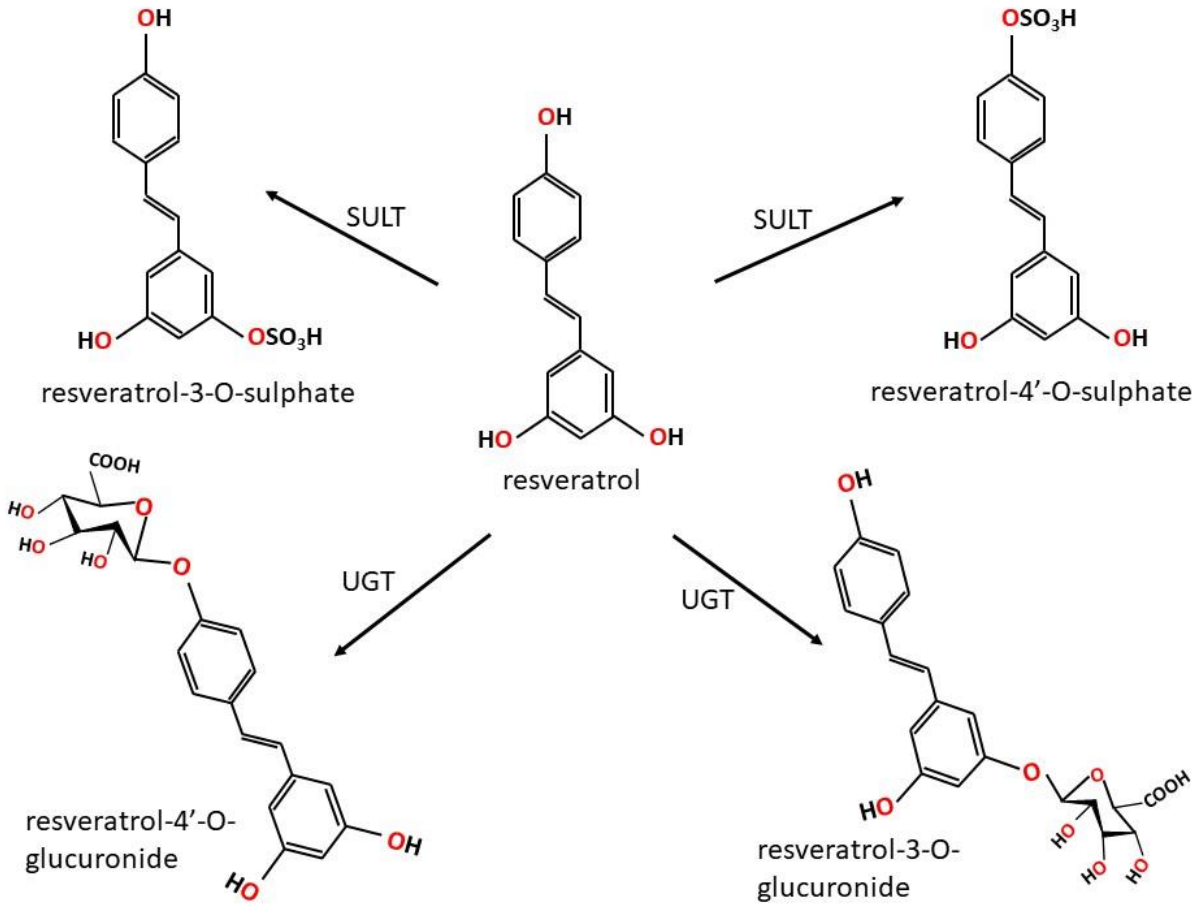



**Figure 5: Metabolic pathways and common metabolites of resveratrol**

In the current study, I selected two resveratrol analogues with different pharmacokinetic profiles (i.e. pterostilbene, with higher bioavailability and gnetol, with lower bioavailability). Comparison of these stilbenoids in animal studies may reveal the effects of bioavailability on the biological activities of these compounds.

### 2.12.5 Stability

In terms of stability, stilbenoids are sensitive to heat, air, light and oxidative enzymes. Specifically, *trans* to *cis* isomerization of resveratrol occurs on exposure to both ultraviolet and visible light.[308] The *trans*- form is known to be the more active form of resveratrol.[309] Furthermore, resveratrol may be degraded by oxidation under certain conditions, for example in the presence of sodium

bicarbonate.[310, 311] In addition to increasing solubility, complexation with cyclodextrin improves the photostability of stilbenoids.[291] Resveratrol was more stable in human plasma than in organic solvents. This is possibly due to binding with plasma protein such as albumin.[312] The solubility and stability of resveratrol and pterostilbene can also be improved by incorporation into liposomes.[292]

### 2.12.6 Safety

Toxicological data show that at low doses, resveratrol is well-tolerated in humans, although safety information following long term administration is still lacking.[313] Furthermore, administration of pterostilbene in a clinical trial at a dose of 125 mg twice daily for 6-8 weeks was found to be safe and did not evoke any remarkable adverse reaction.[314] The safety of gnetol, as a relatively nascent molecule in research, has not been evaluated in humans. Therefore, additional randomized clinical studies with larger population samples and longer follow-up periods are important to further ascertain the safety of resveratrol and other stilbenoids.

## 2.13 Cardioprotective effects of stilbenoids

Interest in the cardioprotective effects of resveratrol was initially stimulated by observation of the French paradox, in which mortality due to coronary heart disease was significantly reduced among the southwestern French population despite deleterious risk factors such as high intake of dietary cholesterol, saturated fat and smoking.[315] A WHO study, Worldwide Monitoring System for Cardiovascular Diseases, revealed lower mortality rates for ischemic heart disease in France compared to other developed countries such as the United Kingdom and United States.[316] Cardioprotection was observed despite the presence of similar risk factors for coronary heart disease such as high blood pressure, high body mass index, and high cholesterol.[316]

The first attempt to explain the French paradox was by Renaud in 1992 in an epidemiological review.[317] Consumption of wine was negatively correlated with CHD mortality.[316] Since there is relatively high wine consumption among the French population, Renaud postulated that the French paradox might be due to the ability of wine consumption to negate the deleterious effects of dairy fat consumption.[317] Thus phenolic constituents of red wine such as resveratrol garnered attention of biomedical researchers. Many studies have shown the beneficial effects of stilbenoids on vascular function, platelet biology, atherosclerosis, oxidative stress, cardiac hypertrophy and ischemic-reperfusion injury as discussed below. Of the three stilbenoids reviewed here, resveratrol is the most widely studied, followed by pterostilbene and gnetol. The cardioprotective effects of resveratrol have been recently reviewed by Zoedoky *et al.* [27]

**Vascular compliance and blood pressure**

Resveratrol (2.5 mg/kg/day for 12 weeks) increased mesenteric small artery compliance and reduced wall stiffness in normotensive Wistar-Kyoto (WKY) rats. Resveratrol treatment also attenuated arterial compliance in the SHR, at least in part through inhibitory actions on pro-growth ERK signaling.[318] The effect of resveratrol on blood pressure in animal models is dose dependent. Resveratrol had no effects on blood pressure at low doses (2.5 mg/kg/day for 10 weeks),[319] whereas administration of higher doses of resveratrol such as 200 mg/kg/day for 4 weeks[320] reduced systolic blood pressure in SHR. Combination of low dose resveratrol (2.5 mg/kg/day) with hydralazine (25 mg/kg/day) was also more effective than resveratrol or hydralazine alone in reducing blood pressure in SHR.[321] Thus, resveratrol may be useful as an adjunct or supplement to current therapies.

Likewise, in humans, a meta-analysis of six randomized control trials showed that resveratrol consumption at a dose of 150 mg/day, but not lower doses reduced systolic blood pressure. Neither

low dose nor high dose resveratrol reduced diastolic blood pressure.[322] To date, there are few studies about the effect of pterostilbene on blood pressure in humans. One randomized double-blinded placebo-controlled trial indicated that high dose pterostilbene (125 mg twice daily) reduced both systolic and diastolic blood pressure while a lower dose of 50 mg twice daily did not.[323]

**Platelet biology**

Among other factors, activation of platelet aggregation is a major contributor to the development of atherothrombosis. In response to rupture of an unstable atherosclerotic plaque, platelets are activated, resulting in thrombus formation.[324] The detached plaque and the resulting thrombus may enter the systemic circulation and cause occlusion of blood vessels, thereby limiting blood supply to organs such as the heart (myocardial infarction) or the brain (stroke). Thus, inhibition of platelet activity is an important strategy to prevent such thrombotic events.[325, 326]

Resveratrol inhibited platelet aggregation in both animal and human studies.[327-329] Furthermore, resveratrol (50 μg/mL) inhibits platelet aggregation induced by collagen, epinephrine, and thromboxane *in vitro*, and these effects may be attributable to suppression of cyclooxygenase (COX)-1 in the arachidonic acid pathway.[330] Other mechanisms that may be involved include inhibition of the MAPK pathway, activation of the nitric oxide/cGMP pathway[331] and inhibition of phosphoinositide signaling.[328]

Similar to resveratrol, pterostilbene also has a strong inhibitory action on platelet aggregation, and stimulates nitric oxide production in platelets.[332] Furthermore gnetol, along with other monomeric stilbenoids (trans-resveratrol and isorhapotigenin) extracted from *Gnetum macrostachyum*, inhibited arachidonic acid-induced platelet aggregation.[333] However, gnetol did not inhibit platelet aggregation induced by thrombin.[333] In this study, gnetol also inhibited platelet-collagen adhesion

in a dose-dependent manner, although to a lesser extent compared to resveratrol. These data are consistent with *in vitro* ELISA assay reported by Remsberg *et al.* where gnetol inhibited COX with a stronger inhibitory action on COX-1 compared to COX-2.[295]

**Ischemia-reperfusion injury**

Resveratrol (100 μM) attenuates ischemia-reperfusion injury in neonatal cardiomyocytes exposed to 2-hour simulated ischemia and 4-hour simulated reperfusion possibly by decreasing intracellular calcium, preventing apoptosis and enhancing activities of ROS scavenging enzymes such as superoxide dismutase.[334, 335] Other mechanisms that might confer anti-oxidant effects of resveratrol reportedly include modulation of the mitochondrial membrane permeability transition pore (mPTP),[336] activation of AMPK,[337] and induction of NOS.[338-340]

In cardiomyocytes, pterostilbene protects against hypoxia-reoxygenation injury via activation and up-regulation of SIRT1.[341] SIRT1 is a NAD+-dependent protein deacetylase that, upon activation, activates PGC-1α via deacetylation and thus improves mitochondrial function and oxidative capacity.[342] Here, pre-treatment of H9c2 cells with splitomycin, a SIRT1 inhibitor, abolished the protective effects of pterostilbene.[341] In an animal model of ischemia-reperfusion injury, pterostilbene improved cardiac function and reduced markers of oxidative stress and inflammation such as TNF-α, IL-1β and myeloperoxidase activity.[343] These data are consistent with results from other groups that showed protective effects of pterostilbene on myocardial ischemia-reperfusion injury via inhibition of apoptosis and attenuation of inflammatory markers in rats.[344, 345] Pterostilbene improved cardiac function and decreased myocardial infarct size in a rat model of ischemia-reperfusion injury.[344] Here treatment with pterostilbene reduced expression of TNF-α and IL-1β, thus reducing cardiac inflammation. Furthermore, pterostilbene inhibited apoptosis by increasing Bcl-2 expression while decreasing Bax expression.[344]

**Atherosclerosis**

Of the five types of lipoproteins, there are two major types that function as carriers of cholesterol in the systemic circulation: high density lipoprotein (HDL) and low density lipoproteins (LDL). High levels of HDL in the circulatory system are considered "good" while high levels of LDL "bad." Due to their low molecular weight, a high level of LDL predisposes to lipid accumulation in the arterial wall, leading to atherogenic processes.[346] Furthermore, LDL oxidation plays an important role in atherogenesis. Oxidized LDL promotes accumulation of inflammatory cells such as macrophages causing build-up of plaques on the vessel wall.[347, 348] Therefore, suppression of LDL oxidation is an important anti-atherosclerotic therapeutic target. Flavonoids from grape juice and red wine, for example, inhibit plasma oxidation of LDL in humans.[349]

Resveratrol modifies vascular function,[318, 350] attenuates lipid accumulation,[351, 352]and modulates gene expression related to lipogenesis and lipolysis.[353] Resveratrol also inhibits oxidized LDL (oxLDL)-induced apoptosis in vascular endothelial cells.[354, 355] Similarly, pterostilbene protects human vascular endothelial cells against apoptosis.[356] Pterostilbene induced cytoprotective autophagy in vascular endothelial cells via activation of AMPK and downstream inhibition of mTOR signaling.[357] Here, pterostilbene activated AMPK via upstream CAMKKβ.[357] Recent studies indicate that pterostilbene attenuates high fat-induced atherosclerosis in mice via suppression of pro-inflammatory cytokines such as TNFα, TGFβ, IL-1β and IL-6 among others.[358] Pterostilbene inhibits proliferation of vascular smooth muscle cells and progression of cell cycle by regulating Akt kinase.[359] Taken together, these research findings suggest protective properties of stilbenoids on the vascular endothelium, which may delay the initiation and progression of atherosclerosis.

**Cardiac hypertrophy**

Resveratrol prevents cardiomyocyte and cardiac hypertrophy in isolated NE-treated cardiomyocytes and in the pressure overload model of cardiac hypertrophy.[24-26] One possible explanation of anti-hypertrophic actions of resveratrol is via activation of NO-AMPK signaling.[24] Resveratrol also inhibited hypertrophy induced by pressure overload (concentric hypertrophy) but not volume overload (eccentric hypertrophy).[360] The proposed mechanism for the effect of resveratrol on pressure overload-induced hypertrophy includes alleviation of oxidative stress and increased NO production via upregulation of endothelial nitric oxide synthase (eNOS).[360] Of note, eNOS level and activity remain unchanged in the volume-overload model of hypertrophy,[361] suggesting that unique signaling pathways are involved in pressure-overload versus volume-overload hypertrophy. Therefore, authors concluded that resveratrol may be used in pressure-overload disease settings such as hypertension and aortic stenosis.[360] Resveratrol also prevented development of hypertrophy in SHR, a genetic model of hypertension and hypertrophy, via the LKB-AMPK-eNOS signaling axis,[319, 362] and grape powder containing polyphenols such as resveratrol, anthocyanins and catechins improved cardiac and vascular function in SHR.[363] The effects of pterostilbene and gnetol on cardiac hypertrophy have not been explored.

**Cardiovascular clinical trials**

Despite the promising effects described above for stilbenoids, the efficacy of specific grape polyphenols such as resveratrol is yet to be established in humans. For example, administration of resveratrol (7 mg/day for 12 weeks; n=15) to non-obese healthy women did not change metabolic parameters such as insulin sensitivity, mitochondrial function, AMPK signaling and inflammatory markers.[364] The absence of effect of resveratrol on metabolic parameters in normal subjects is consistent with earlier results obtained in normal rodents.[365] Thus, we may infer that resveratrol

exerts its effects only in metabolic disease conditions such as obesity, type II diabetes and dyslipidemia.

Effects of resveratrol administration on primary and secondary prevention of CVD have shown some promise,[366-368] but small sample size (n=75) and short follow-up (1 year) limit the clinical relevance of the promising outcomes. In this study, 75 subjects were randomly divided into 3 groups. One group was administered resveratrol-containing grape extract capsules (8.1 mg/d for the first 6 months and 16.2 mg/d for the next 6 months), another group was given grape extract capsules containing no resveratrol, and the third group was given placebo capsules. All subjects in this study were also on statins and were treated according to the prevailing guidelines for primary prevention of CVD.[367] At the study endpoint, patients treated with resveratrol-containing grape extract showed improved inflammatory and fibrinolytic status compared to placebo and grape-extract only group.[367] Since resveratrol provided additional benefits in patients with high risk of CVD, over and above other polyphenols in the grape extract, resveratrol may complement current guidelines for the primary prevention of CVD.[366, 367]

Although resveratrol and pterostilbene exerted antihyperlipidemic actions in animal models,[369, 370] clinical trials in humans do not show reduction in LDL/HDL ratio. In a meta-analysis of randomized control trials, supplementation with resveratrol did not significantly affect lipid parameters such as total cholesterol, LDL, HDL and triglyceride levels.[371] Both high and low doses of pterostilbene increased LDL and had no effect on HDL and triglycerides in a randomized placebo-controlled trial.[323] Administration of grape extract did not increase LDL in this study.

To determine the effect on secondary prevention of CVD, resveratrol-containing grape extract (8.1 mg/d for the first 6 months and 16.2 mg/d for the next 6 months) was administered to patients with stable coronary artery disease in addition to their regular medications and dietary restrictions.[368]

Compared to the placebo and grape extract only group, there was an increase in anti-inflammatory adiponectin and a decrease in thrombogenic plasminogen activator inhibitor type 1 (PAI-1) in the group that received resveratrol.[368] Thus, resveratrol may exert its cardioprotective effects by improving anti-inflammatory response and preventing atherothrombotic signaling.[368]

## 2.14 Other biological effects of stilbenoids

**Diabetes**

Diabetes is a chronic metabolic disease associated with inflammation and oxidative stress. Due to its anti-inflammatory and antioxidant effects, resveratrol can mitigate the development of diabetic complications. Preclinical data show that resveratrol might be beneficial in the management of diabetes by improving insulin resistance, improving defective insulin signaling, preventing pancreatic beta cell apoptosis and dysfunction.[372] Resveratrol prevents hyperglycemia in diabetic animal models by increasing glucose uptake and translocation of GLUT 4 to the membrane.[373] Furthermore, resveratrol improved glucose tolerance and reduced the expression of advanced glycated end-products (AGE) receptors in diabetic rat liver and kidney.[374, 375] Resveratrol prevents production of ROS and reactive nitrogen species such as superoxide anion ($O2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide and malondialdehyde (MDA) while increasing levels of antioxidant enzymes such as SOD, catalase and glutathione peroxidase in diabetic animals.[376] Resveratrol also inhibits the pro-inflammatory marker nuclear factor κB (NFκB), and thus reduces the production of inflammatory markers such as TNF-α, IL-1β, IL-4, and IL-6.[376] In addition, resveratrol increased insulin sensitivity, glucose tolerance and mitochondrial biogenesis in an AMPK-dependent manner.[377] In this study, resveratrol was unable to elicit the same effect in AMPK-deficient mice suggesting the role of AMPK in the metabolic actions of resveratrol.[377]

While some human studies have shown the benefits of resveratrol on glycemic control, other studies did not demonstrate significant effects. Administration of resveratrol (250 mg/day for 30 days) along with metformin or glibenclamide improved glycemic parameters in type 2 diabetes patients.[378] Movahed and colleagues also reported that resveratrol (1 g/day for 45 days) reduced fasting blood sugar, HbA1c and systolic blood pressure.[379] Indeed, a much lower dose of resveratrol (5 mg/day for 28 days) reduced HbA1c, systolic blood pressure and improved insulin sensitivity but did not affect the homeostatic model of assessment of insulin resistance (HOMA-β).

In contrast, in a recent randomized control trial by Thazhath *et al.,* administration of 500 mg of resveratrol twice daily for 5 weeks in diet-controlled type 2 diabetes did not significantly improve glycemic control.[380] There was no difference between the fasting glucose level, postprandial glucose level, HbA1c, gastric emptying and glucagon-like peptide 1 secretion in the resveratrol-treated versus the placebo group. Similarly, resveratrol treatment for 6 months did not improve metabolic parameters in type 2 diabetic patients.[381] Therefore the effect of resveratrol on diabetes in human is not fully understood.

Pterostilbene improves glycemic control in insulin-resistant obese rats by increasing hepatic glucokinase activity and increasing skeletal muscle glucose uptake.[382] *In vitro* studies also indicate that pterostilbene protected pancreatic beta cells against oxidative stress and apoptosis.[383] Antihyperglycemic properties of pterostilbene along with other phenolic constituents of *Pterocarpus marsupium* have been reported.[384, 385] Whereas pterostilbene has been shown to be beneficial in animal models of diabetes and metabolic disorder, human data are still limited. The effect of pterostilbene in type 2 diabetes in humans is yet to be explored. Administration of blueberry (*Vaccinium myrtillus*) and sea buckthorn (*Hippophae rhamnoides*) extract to children

with type 1 diabetes for 2 months elicited a reduction in HBA1c levels and an increase in SOD and glutathione peroxidase levels. This effect may be due to the presence of antioxidants and polyphenols including pterostilbene in the extract.[386]

**Neurodegeneration**

The neuroprotective effects of stilbenoids are mostly due to their anti-oxidant and anti-inflammatory properties.[387-390] Neurodegenerative disorders such as Parkinson's and Alzheimer's diseases are associated with oxidative stress and mitochondrial dysfunction leading to loss of function and death of neurons.[391] Resveratrol protects neurons against ROS and improved motor co-ordination in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in mice by scavenging hydroxyl radicals.[392] Resveratrol also protected against lipopolysaccharide-induced dopaminergic neurodegeneration via inhibition of microglial activation and NFkB signaling in the microglial.[393] Alzheimer's disease is characterized by development of plaques made up of amyloid-beta protein in the hippocampus and cerebral cortex. Aggregation of amyloid-beta plays a key role in the pathogenesis of Alzheimer's disease.[394] There is also evidence that amyloid-beta contributes to oxidative damage in the neurons by inducing lipid peroxidation, protein oxidation and DNA oxidation.[395] Resveratrol holds therapeutic potential in the treatment of Alzheimer's disease due to its ability to reduce levels of amyloid-beta. In a study by Marambaud *et al*., although resveratrol did not inhibit the production of amyloid-beta, it promoted proteasome-dependent degradation of amyloid-beta.[396]

Pre-treatment with resveratrol protects against cerebral ischemia-reperfusion injury in rats.[397] Levels of nuclear factor erythroid 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) were upregulated in the resveratrol-treated group.[397] Infarct volume and brain water content were reduced and neurological scores were improved by resveratrol pre-treatment. Resveratrol protected

against neuronal death in a rat model of global cerebral ischemia via activation of PI3K-Akt signaling and downregulation of glycogen synthase kinase-3β (GSK-3β) and cAMP response element-binding protein (CREB).[398] Apart from effects on cerebral ischemia, resveratrol improved cognition in an animal model of vascular dementia.[399] Here, vascular dementia was induced by bilateral occlusion of the common carotid arteries for 8 to 12 weeks. Treatment with resveratrol improved learning and memory scores. The lipid peroxidation product, malondialdehyde, was reduced while levels of antioxidant enzymes such as superoxide dismutase and glutathione were increased in the hippocampus and cerebral cortex of the resveratrol-treated group.[399] This suggests the antioxidant role of resveratrol in the neuroprotective effects.

Although reports on the effects of pterostilbene on specific animal models of Alzheimer and Parkinsonism are limited, effects in cell culture models of neurotoxicity have been reported. Pterostilbene exerts neuroprotective effects against high glucose-induced injury in neuroblastoma cells.[387] Here, pterostilbene prevented cell death and generation of ROS in a dose-dependent manner. Pterostilbene also increased activities of mitochondrial complexes I and III, mitochondrial cytochrome C, and mitochondrial membrane potential. In addition, the levels of Nrf2, HO-1 and glutathione S-transferase (GST) were elevated with pterostilbene treatment.[387] Similarly, pterostilbene ameliorates glutamate-induced neuronal oxidative stress via Nrf2 signaling.[388] In a recent study, memory deficit induced by streptozotocin in rat brain was improved by pterostilbene treatment.[400] Pterostilbene also improves cholinergic transmission via inhibition of cholinesterases.[400] Studies on the effect of gnetol on the nervous system are scarce. There is one report that gnetol reversibly and competitively inhibits butyryl cholinesterase, and this may be applicable in the treatment of Alzheimer disease.[401]

In a randomized placebo-controlled clinical trial, resveratrol, at a dose of 500 mg per day gradually escalated to 1000 mg twice daily for 52 weeks, was found to be safe and well-tolerated in Alzheimer disease patients.[402] In this study, resveratrol prevented reduction of cerebrospinal fluid and plasma levels of amyloid-beta 40 (the most abundant amyloid beta isoform) compared to the placebo group, but did not affect several other important Alzheimer disease biomarkers.[402] Thus the results of this study did not clearly indicate benefit of resveratrol in Alzheimer disease. In contrast, supplementation with 200 mg/day resveratrol and 320 mg/day quercetin (to improve bioavailability of resveratrol) for 26 weeks improved memory performance in healthy overweight elderly subjects.[403] A more recent clinical trial also indicated that resveratrol improves cognition, mood and cerebrovascular function in postmenopausal women when administered at a dose of 75 mg twice daily for 14 weeks.[404] Resveratrol improved cerebral blood flow by dilating cerebral blood vessels and thus improved cognitive performance in type 2 diabetic patients.[405, 406] In conclusion, results of human studies show that resveratrol improves memory function and cognition in healthy subjects and diabetic patients with sub-clinical cognitive impairment but not in Alzheimer disease *per se*. The effects of pterostilbene and gnetol on memory and cognition are yet to be studied in humans.

**Obesity**

Obesity is characterized by excessive adipose tissue caused by increased caloric intake and/or inadequate energy expenditure. As defined, body mass index greater than 30 and waist circumference greater than 88 cm and 94 cm in women and men respectively[407] is one of the major risk factors for the development of CVD. Weight management is part of the typically recommended lifestyle modifications. Resveratrol inhibits adipogenesis, attenuates lipid accumulation and increases lipolysis in mature adipocytes.[408] The lifespan of mice fed high caloric

(60% fat) diets supplemented with 0.04% resveratrol was lengthened.[409] Furthermore, administration of 150 mg/day trans-resveratrol over 30 days mimics the effect of caloric restriction in obese humans and thus may help to control obesity and other metabolic syndromes.[410] Similarly, resveratrol delayed age-related abnormalities, albeit without significant effect on longevity in non-obese mice.[411] This indicates that resveratrol may help counter the adverse metabolic effects of obesity. In non-human primates, resveratrol was shown to suppress body mass gain, increase metabolic rates and total energy expenditure.[412, 413]

Since resveratrol activates AMPK, and AMPK is involved in energy balance regulation and mitochondrial biogenesis, AMPK was proposed as a possible mechanism by which resveratrol protects against metabolic dysfunction.[377] In AMPK-deficient mice, resveratrol failed to improve insulin sensitivity possibly due to non-effect on mitochondrial content and fatty acid oxidation in skeletal muscles. This caused a build-up of lipids that may inhibit insulin action.[377] Furthermore, resveratrol activates sirtuins.[414] In high fat-fed mice, resveratrol improved glucose tolerance by enhancing sensitivity to insulin, while protecting the mice against development of obesity.[415] This beneficial effect of resveratrol was lost when the acetylation sites of PGC-1α were mutated or when SIRT expression was disrupted using SIRT-deficient mice, thus indicating the role of PGC-1α /SIRT signaling.[415]

Similar to resveratrol, pterostilbene exhibits anti-obesity properties. Pterostilbene reduces fat accumulation in adipose tissue by inhibiting lipogenesis, while enhancing fatty acid oxidation in the liver.[416] Pterostilbene also increased AMPK and acetyl-coA carboxylase activities in adipose tissue. Furthermore, in Zucker *fa/fa* rats, pterostilbene increased thermogenesis in brown adipose tissue by increasing the gene expression and translation of uncoupling protein 1 (UCP-1), a key mediator of thermogenesis.[417] Another recent study showed that pterostilbene reduced

accumulation of abdominal white adipose tissue, increased fat metabolism and suppressed lipogenesis in obese rats.[418] Here, mRNA levels of UCP1 were also increased while mRNA levels of fatty acid synthase and leptin were reduced.[418] While effects on obesity have been reported for both resveratrol and pterostilbene as discussed above, the effect of gnetol on obesity is yet to be explored.

Despite promising effects of resveratrol on obesity in animal models, a systematic review of nine randomized controlled trials on the effects of resveratrol on obesity in humans showed limited evidence for the use of resveratrol in obesity and weight management.[419] Most studies did not find a reduction in body weight after treatment with resveratrol for a period of 4 to 12 weeks in obese and non obese subjects.[364, 410, 420, 421] The only study that reported a clear beneficial effect on body weight administered resveratrol 500 mg three times per day for 12 weeks in obese subjects with metabolic syndrome.[422] Thus, it may be concluded that a period of at least 12 weeks is required for resveratrol to elicit anti-obesity effects in obese humans.[423] Further human studies are required to confirm the effects of resveratrol, and the results of ongoing clinical trials may provide more information in this regard.

**Cancer treatment and prevention**

Interventions to prevent and/or treat cancer may occur at specific stages of carcinogenesis such as initiation, promotion and progression. Stilbenes block metabolic activation of pro-carcinogens by inhibiting specific isoforms of cytochrome P450 (CYP) enzymes and thus prevented the initiation of carcinogenesis in cultured human tumor cells.[424, 425] Moreover, resveratrol induced activities of phase II metabolizing enzymes such as uridine-5-diphospho (UDP) glucuronyltransferase and NADPH:quinone oxidoreductase in mouse epidermis.[426, 427] Resveratrol may prevent the progression of cancer by suppressing actions of transcription factors and growth factors involved

in the initiation and promotion of cancer in cell culture studies.[428] Furthermore, resveratrol and pterostilbene prevent proliferation and induce apoptosis in various cancer types including breast, prostate, pancreatic, liver and colorectal cancer, recently reviewed by Carter *et al*.[429, 430] Mechanisms that have been implicated in the anticancer properties of resveratrol and pterostilbene include induction of apoptosis, inhibition of proliferation, cell cycle arrest and inhibition of angiogenesis.[431, 432] Pterostilbene also exerts more potent inhibitory actions on human colon cancer cells than resveratrol due to its higher lipophilicity.[433] Although data from animal studies are mostly positive, there is limited human clinical data to support the use of stilbenoids in the treatment and management of cancer. Thus, more studies are needed in this area before resveratrol and other stilbenoids can be used in the human cancer treatment or prevention.

**Depigmentation**

Melanin, a pigment which protects the skin against harmful effects of ultra-violet radiation, is produced by melanocytes in the process of melanogenesis.[265] Overproduction of melanin in some acquired hyperpigmentation disorders may cause some skin concerns.[434] Furthermore, deregulation of melanogenesis has been associated with more aggressive progression of melanotic melanomas, due to production of immunosuppressive intermediates.[435, 436] Thus, inhibition of melanogenesis may serve as an adjuvant therapy in the treatment of melanomas.[437] Melanogenesis is a multistep process which begins with conversion of L-tyrosine to L-DOPA, the rate limiting step catalyzed by the enzyme tyrosinase.[265] Gnetol inhibited melanin production in murine B16 melanoma cells via inhibition of tyrosinase.[286] Similarly, resveratrol inhibited ultraviolet B-induced hyperpigmentation in guinea pig skin.[438] Here, resveratrol also downregulated melanogenesis-related proteins such as tyrosinase, TRYP1, TRYP2 and MITF in melanoma cells.[438] There is evidence to show that pterostilbene is a more potent inhibitor of melanogenesis

than resveratrol in α-melanocyte stimulating hormone (MSH)-stimulated B16/F10 melanoma cells.[439] Moreover, pterostilbene inhibited tyrosinase enzyme activity in a dose-dependent manner.[439] The ability of stilbenoids to inhibit melanin synthesis make them suitable candidates for use in the cosmetic industry to treat acquired hyperpigmentation disorders and possibly as adjuvants in the treatment of melanotic melanomas.

## 2.15 Animal models of hypertrophy and heart failure

Many studies on the cardiovascular system employ isolated cultured cardiomyocytes from neonatal or adult rats. This allows characterization of cell properties without the influence of homeostatic mechanisms that occur in whole animal models. *In vitro* cell culture does not completely represent conditions of a whole heart pumping system. Thus, after physiologic and pharmacologic mechanisms have been established in cell culture experiments, it is important to scale up to whole animal studies by using appropriate animal models. There are many animal (rodent) models of hypertrophy, hypertension and heart failure, some of which overlap in their applications. For instance, SHR develop high blood pressure at a young age and later develop left ventricular hypertrophy.[440] The spontaneously hypertensive heart failure (SHHF) rat also develops high blood pressure early in life, which then progresses to left ventricular hypertrophy. However, male SHHF-obese rats develop heart failure at a younger age of 10-12 months old compared to 18 months in SHR.[441] Hypertrophy may also be induced in rats by pressure-overload (aortic constriction),[442] volume-overload (aortic regurgitation)[443] and administration of vasoactive compounds such as Ang II, NE and isoproterenol.[362, 444, 445]

Pressure overload conditions can be achieved in animals by surgical constriction of the aorta, a procedure performed under anesthesia. In rats, left ventricular hypertrophy is induced by banding of the ascending aorta.[442] The procedure is relatively easy and has a low mortality rate of 10-15%.[442] This model has been widely used to study structural and functional changes in ventricular hypertrophy. However, the pitfall of this model is the acute nature of the disease model. It does not completely mimic the gradual progression of pressure overload that occurs in human clinical cases. Furthermore, transverse aortic constriction (TAC) is used in mice to induce hypertrophy and heart failure.[446] The extent of aortic constriction determines the extent of hypertrophy and

onset of heart failure. Aortic constriction can also be achieved by constriction of the abdominal aorta.[25, 447] Although pressure overload animal models are primarily used to study cardiac hypertrophy, these models also progress to heart failure in some cases.[448] The major contributor for the development of heart failure was found to be the type of ventricular remodeling that accompanies pressure overload rather than myocardial dysfunction.[448] Eccentric hypertrophy was observed in animals that progress to heart failure, while concentric hypertrophy occurred in animals that do not exhibit heart failure.[448]

The SHR is a model of hypertension and hypertrophy. It is the most commonly used genetic hypertensive animal model and has been employed in research for the past 50 years.[449] SHR was originally discovered in Japan by mating an outbred male WKY rat with marked elevation of blood pressure and a female with slightly elevated blood pressure.[449] The descendants of this colony were further bred and developed in the United States into what we know today as SHR. Most SHR colonies develop pre-hypertension at age 6-8 weeks. This progresses into full blown hypertension at age 18-22 weeks. About 57% of SHR also progress to heart failure in the last few months of their lives.[450] Since SHRs were originally bred from WKY rats, WKY rats are used as the normotensive controls. Advantages of the SHR include similarities with progression of hypertension in humans.[451] Moreover, drugs that reduced blood pressure in SHR have been shown to also lower blood pressure in humans. One limitation of this model is that the specific gene or groups of genes responsible for the development of hypertension in these animals are yet to be identified, although there have been many advances in genome mapping and sequencing of the SHR.[452]

The SHHF rat, which was developed after the SHR, is a model of heart failure that superimposes hypertension on predisposition to develop heart failure. SHHF is a congenital model of dilated

cardiomyopathy that progresses into decompensation and heart failure more quickly than SHR.[441] SHHF rats were first obtained by crossbreeding the SHR rat with the SHR-obese rat.[453] The SHR-obese rats were generated by mating an SHR female rat with a normotensive Sprague-Dawley (SD) male rat, thus the use of normotensive SD rats as controls in SHHF rat studies. There are 2 strains of the SHHF model: obese or lean. About 25% of SHHF rats are obese, while the rest are lean.[453] Both SHHF-obese and SHHF-lean phenotypes develop heart failure but at different ages.[454] SHHF-obese rats develop fatal cardiomyopathy at 10-12 months for males and at 14-16 months for females, and die at an earlier age than their lean counterparts. SHHF-lean rats develop hypertension and left ventricular hypertrophy at age 3-5 months and heart failure at about 16-20 months.[453] In contrast to age-matched SHRs, which exhibit concentric hypertrophy, SHHF-lean rats exhibited eccentric hypertrophy at 10-20 months of age, indicating congestive dilated cardiomyophathy.[441]

Heyen *et al.* (2002) examined the structural, functional and molecular characterization of 4 to 18-month old SHHF-lean rats[441] and identified similar features between the progression of heart failure in humans and in the SHHF-lean rat model. SHHF-lean rats exhibited elevated blood pressure as early as age 4 months.[441] Echocardiographic analyses showed decreased relative wall thickness and higher end-diastolic and end-systolic volumes at age 15 and 18 months, indicating dilated hypertrophy.[441] Significant systolic dysfunction, as indicated by reduced fractional shortening and ejection fraction, was also recorded at the same time points. Structural changes included myocardial fibrosis and myocyte degeneration. Markers of inflammation such as infiltrates of monocytes and macrophages were observed, and elevated levels of IL-6, IL-1β and COX2 were detected starting from 9 months of age.[441] This indicates that SHHF-lean rats could

be employed as an experimental model to understand the pathophysiology of human heart failure, while avoiding the marked obesity and insulin resistance phenotypes present in SHHF-obese rats.

## 2.16 Hypothesis and objectives

I hypothesized that stilbenoid polyphenols (resveratrol, gnetol and pterostilbene) preserve cardiac function and prevent cardiac hypertrophy via modulation of AMPK and MITF signaling.

**Objectives**

The cardioprotective effects of gnetol and pterostilbene were queried in isolated neonatal rat ventricular cardiomyocytes and the SHHF rat model. The aims of this project were to determine the:

1. effects of gnetol and pterostilbene on ET-1-induced hypertrophy in isolated neonatal rat ventricular cardiomyocytes;
2. downstream signaling mediators of stilbenoid effects in isolated cardiomyocytes, with emphasis on AMPK and MITF;
3. effects of gnetol, pterostilbene and resveratrol on left ventricular hypertrophy and cardiac function in the SHHF rat model.

*Objective 1: to determine the effects of gnetol and pterostilbene on cardiomyocyte hypertrophy:*

**Rationale**: While resveratrol has been researched and its cardioprotective effects have been widely reported,[24, 26, 455] the activities of other stilbenoids have not been carefully studied. Some of the challenges associated with the use of resveratrol include its poor bioavailability and photostability. Among the stilbenoids, pterostilbene has a much greater bioavailability of 80%[297] compared to 20-38.8% for resveratrol.[29] Furthermore, exposure of resveratrol to UV light causes trans-cis isomerization which largely reduces its biological activity.[308] Gnetol is already widely used in some systems of traditional medicine despite the lack of scientific evidence to support its use. There is therefore a need to probe the biological activities of gnetol and pterostilbene and compare

their effects to resveratrol. The effects of gnetol and pterostilbene on cardiomyocyte hypertrophy have not been previously explored. The first aim of this project was to determine the effects of gnetol and pterostilbene on cardiomyocyte hypertrophy *in vitro*.

*Objective 2: to determine downstream signaling mediators of stilbenoid effects with emphasis on AMPK and MITF:*

**Rationale (i)**: Activation of AMPK *in vitro* inhibits cardiomyocyte size augmentation, protein synthesis, and hypertrophic gene expression.[232, 245] Since resveratrol is known to prevent hypertrophy partly through activation of AMPK,[24, 26, 456] and pterostilbene also activates AMPK in prostate cancer cells,[457] AMPK was selected as a possible signaling effector involved in the antihypertrophic effects of gnetol and pterostilbene.

**Rationale (ii)**: Although MITF has been mostly studied in melanocytes, recent studies indicate the role of MITF-H, the cardiac isoform, in cardiac hypertrophy.[267] Tshori *et al.* demonstrated that MITF is required for the hypertrophic response to β-adrenergic stimulation.[267] Another group established a similar role for MITF in hypertrophic responses as MITF knockdown caused a significantly blunted hypertrophic response induced by Ang II.[268] In non-cardiac cells, ET-1 up-regulates tyrosinase and induces the phosphorylation of MITF.[458, 459] Tyrosinase, a key enzyme involved in melanin synthesis, is one of the downstream targets of MITF,[460, 461] and interestingly, members of the stilbenoid group of compounds have been shown to inhibit tyrosinase.[286, 462] Thus we predict that gnetol and pterostilbene may downregulate MITF and prevent ET-1 induced phosphorylation of MITF. To our knowledge, this is the first study of the effects of stilbenoids on MITF signaling performed in cardiomyocytes. Thus, this novel mechanism was probed using resveratrol as well.

**Rationale (iii)**: In cardiomyocytes, hypertrophy-inducing agents such as ET-1 have been shown to activate the MEK-ERK signaling pathway.[463] MEK-ERK in turn activates MITF via phosphorylation at serine 73 and 409 (at least in melanocytes).[259, 260] ET-1 upregulates tyrosinase and induces MITF phosphorylation in melanocytes.[458, 459] Activation of AMPK may result in inhibition of the MAPK-ERK signaling pathway.[273] Since stilbenoids activate AMPK, it is possible that stilbenoids downregulate MITF via activation of AMPK in the presence of hypertrophic stimuli. Figure 6 depicts how AMPK may cross-talk with MITF signaling via the MEK-ERK pathway in the presence of hypertrophic stimuli in cardiomyocytes.

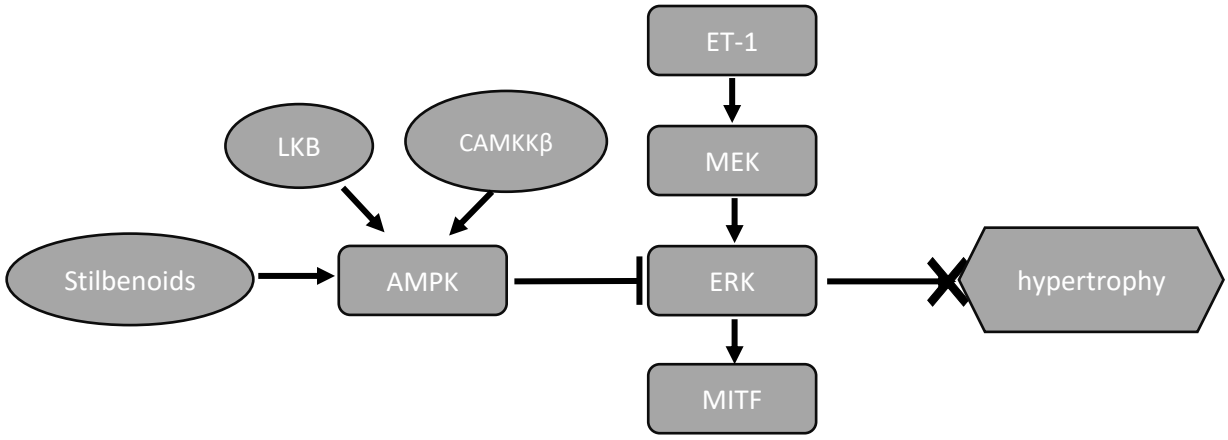



**Figure 6: Proposed AMPK-MITF signaling in cardiomyocytes**

*Objective 3: to determine effects of gnetol and pterostilbene on cardiac function and hypertrophy in vivo:*

**Rationale**: After establishing the anti-hypertrophic effects of gnetol and pterostilbene in cardiomyocytes *in vitro*, their effects on cardiac hypertrophy *in vivo* were examined in comparison to resveratrol using the SHHF rat model. Since resveratrol inhibited cardiac hypertrophy at a dose of 2.5 mg/kg/d in aortic-banded rat model of hypertrophy,[25] I used equivalent doses of gnetol and

pterostilbene, administered by oral gavage for 8 weeks, and compared their effects to that of resveratrol. Furthermore, levels of AMPK and markers of fibrosis (collagen I and smooth muscle actin [SMA]) in left ventricular tissue were also examined.

# CHAPTER 3

# MATERIALS AND METHODS

# 3.0 CHAPTER THREE: MATERIALS AND METHODS

## 3.1 Materials

Gnetol was synthesized and donated by Sabinsa Corporation (New Jersey, USA). Resveratrol, pterostilbene, ethanol, endothelin-1, compound C, polybrene, α-actinin antibody, Alexa Fluor goat anti-mouse secondary antibody, β-actin antibody and cOMPLETE protease inhibitor cocktail were obtained from Sigma-Aldrich (Oakville, Canada). Antibodies against AMPK and phosphorylated AMPK were purchased from Cell Signalling (Whitby, Canada). Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay kit and calcein AM were from Molecular Probes/Invitrogen (Burlington, Canada). The RNA extraction kit was from Qiagen while PVDF membrane was from BioRad (Mississauga, Canada). DMEM high glucose media, cosmic calf serum and iron-supplemented bovine calf serum were from ThermoFisher Scientific (Mississauga, Canada). Triton X-100 was from EMD Millipore (Billerica, USA). Scrambled lentiviral sequences and lentiviral shRNA against AMPK α1-1, α1-2, α2-1 and α2-2 were obtained from the lentiviral core service of the University of Manitoba.

Other instruments and equipment include the following: inverted fluorescent microscope (Olympus 1X81, Markham, ON, Canada), medical film processor (Konica SRX-101A, Taiwan), electrophoresis power supply (Biorad Powerpac Basic, Mississauga, ON, Canada), microplate reader (Fluostar Omega, BMG labtech, Offenburg, Germany), and incubator (ThermoForma direct heat CO2 incubator).

## 3.2 Neonatal rat ventricular cardiomyocyte isolation and culture

Neonatal rat pups were produced from a larger in-house Sprague-Dawley rat-breeding colony. The pups were born in an aspen bedding-enriched polycarbonate rat cage suspended in a racking system that forms the cage lid.

Neonatal rat ventricular cardiomyocytes were isolated from the ventricles of 60 neonatal (24-48 h old) Sprague-Dawley rats per preparation as previously described.[464] Briefly, each pup was decapitated and the heart excised and washed in calcium- and bicarbonate-free HEPES-buffered Hanks' (CBFHH) solution. After removing the atria and aorta, ventricles were minced into small pieces. The minced tissue was then digested in trypsin and DNase I at 37°C and mechanically disrupted. After each 5 to 7-minute cycle of digestion, the suspended cells were removed and placed in bovine calf serum (BCS) and centrifuged at 394 x g, at 4°C for 5 min. After centrifugation, the supernatant was discarded and sediment re-suspended in DMEM containing 10% cosmic calf serum (CCS) and antibiotics. Cardiomyocytes were then separated from other cell types such as fibroblasts, endothelial cells and vascular smooth muscle cells by pre-plating for 1 hour at 37°C in tissue culture plates. Cells that do not attach firmly to the plates within 1 hour are cardiomyocytes. Following the 1 hour period of pre-plating, suspended cardiomyocytes were recollected and plated at appropriate densities in gelatin-coated plates for each experiment and cultured at 37°C in incubator with 5% $CO_2$. After 18-24 h, cells were serum-deprived by changing to DMEM media with 0% CCS and antibiotics for another 24 h to make them quiescent and prevent over-growth. Treatments were started after the period of serum deprivation.

## 3.3 Viability tests

Calcein AM staining was used to determine the viability of cells. Cells were treated with 1, 5, 10, 50 and 100 µg/mL of gnetol and 1, 5, 10, 50 µg/mL pterostilbene. After treatment for 24 h, the

media was removed, and 400 µl 3 µM calcein AM in warm PBS was added to each well (24-well plate, cell density 350,000 cells/well) and incubated for 30 min at 37 °C in the dark. The following were used as controls: (i) wells containing no cells + solution of 3 µM calcein AM in PBS to determine background signal, (ii) wells containing cells treated with vehicle to serve as live controls, and (iii) wells containing cells treated with 0.2% Triton X-100 for 15 min to determine the background signal for dead cells (dead control). Calcein fluorescence was measured using the fluorescent plate reader at excitation/emission wavelengths of 485 nm/535 nm.

## 3.4 Treatments

Isolated neonatal ventricular cardiomyocytes were pre-treated with resveratrol (1-100 µg/mL), gnetol (1-100 µg/mL) and pterostilbene (1 µg/mL) for 1 hour before being challenged with endothelin-1 (ET-1; 0.1 µM, 24 h). ET-1 is a GPCR agonist that is used to induce hypertrophy in cardiomyocytes.[465] Cells were treated with an AMPK inhibitor (compound C; 1 µM) for 1 hour before treatment with resveratrol, pterostilbene or gnetol. Compound C is also known as dorsomorphin (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine).

## 3.5 Immunostaining and cell size measurements

After treatments, cells were washed two times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 1 hour. After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS and blocked in a blocking solution of 2% non-fat skimmed milk in 0.1% Triton X-100. Cardiomyocytes were then incubated with anti-α-actinin as the primary antibody (1 in 1,000 diluted in the blocking solution) overnight at 4°C and then in Alexa Fluor goat anti-mouse secondary antibody (1 in 700 dilution) for 1 hour at room temperature. Cells were washed with PBS three times for 5 min after each antibody staining, and then viewed under a fluorescent microscope where images were captured for cell size measurement. 6-8 images of non-overlapping

views were captured for each treatment group. The surface areas of 40-45 cells per treatment group were measured by computer-assisted planimetry using ImageJ software (version 1.46r).

## 3.6 RNA extraction and polymerase chain reaction

Myocytes were cultured in 12-well plates ($1\times10^6$ cells/well). After treatment, total RNA was extracted from the cells using a Qiagen RNeasy mini kit as per kit instructions. The extracted RNA was then quantified by optical absorption spectrophotometry. Induction of the fetal gene program, i.e. BNP gene expression, was determined by a one-step qPCR method using forward and reverse primers and iTaq Universal SYBR Green One-Step Kit. BNP primers were as follows: sense, 5'CAGCTCTCAAAGGACCAAGG3' and antisense, 5'CGATCCGGTCTATCTTCTGC3'. GAPDH was employed as the internal control (sense: CTCATGACCACAGTCCATGC and antisense: TTCAGCTCTGGGATGACCT).

## 3.7 Protein synthesis

*De novo* protein synthesis was measured using the Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay kit (Invitrogen). This assay detects the level of incorporation of AHA, which is an analog of the amino acid methionine, into nascent protein synthesized during an incubation period following treatment with gnetol and pterostilbene (with or without ET-1). Cells were plated in a 48-well plate. After treatments, cells were incubated in a methionine-free media containing 50 µM AHA for 2 h. After the 2-hour period, the cells were washed, fixed and permeabilized. Nascent protein content was detected by a click reaction with Alexa Fluor-488 alkyne. Fluorescence was measured by a plate reader at excitation and emission wavelengths of 495 nm and 519 nm respectively. The level of fluorescence corresponds to the level of protein synthesis.

## 3.8 Effects of stilbenoids on AMPK activation and expression

Neonatal cardiomyocytes were cultured in 6-well plates ($2\times10^6$ cells/well). Time-course activation of AMPK by gnetol and pterostilbene was determined by treating cells with gnetol (5 µg/mL) or pterostilbene (1 µg/mL) for 0, 4, 8 and 24 h. After each time point, cells were harvested into RIPA buffer and centrifuged. Conventional western blotting was used to detect the levels of AMPK and phosphorylated AMPK (Thr-172) in cell lysates using β-actin as the loading control.

## 3.9 Lentiviral preparation and infection

After isolation, cells were allowed to grow in DMEM with 10% CCS to about 70% confluence. Cells were then washed 2 times with PBS before the transduction procedure. DMEM without antibiotics was used for transduction. Based on the virus titre for the lentivirus batch used, 20 µl of the virus per mL of media was added to the cells. Polybrene (8 µg/mL) was added to improve virus uptake efficiency into the cardiomyocytes.

Scrambled lentiviral sequences and lentiviral shRNA against AMPK α1-1, α1-2, α2-1 and α2-2 sub-isoforms were utilized. The cells were incubated with the virus for 18 h after which the media was changed to DMEM containing 0% CCS. Media was changed and cell conditions monitored daily. Adequate gene knockdown was detected by western blotting.

## 3.10 Adult rat ventricular cardiomyocytes isolation and culture

Adult male Sprague-Dawley rats (200-250 g) were anesthetized with 3% isoflurane and injected with heparin into the saphenous vein (1000 U/mL at 1 mL/Kg body weight). The heart was immediately removed, placed into a perfusion chamber, cannulated through the aorta, and washed of blood with calcium-free buffer (mM: NaCl 90, KCl 10, KH2PO4 1.2, MgSO4. 7H2O 5.0, NaHCO3 15, taurine 30, glucose 20, pH 7.4) for 5 min. It was then perfused for 20 min (at 37°C)

with calcium-free buffer containing 179 U/mL collagenase II. After perfusion, ventricles were removed, minced and incubated for five min at 37°C with re-circulated collagenase buffer for further digestion. Isolated cardiomyocytes were then plated on coverslips pre-coated with laminin (10 μg/mL) and maintained for 2 h at 37°C and 5% CO2 in medium 199 containing 5% fetal bovine serum, 5% horse serum, and 1% penicillin/streptomycin. After 2 h, the medium was replaced with medium 199 supplemented with 5 mM taurine, 2 mM L-carnitine, 1 mM creatine, 2 μM insulin, and 100 IU/mL penicillin/streptomycin.

## 3.11 Measurements of cardiomyocyte contractility

Contractile properties of adult rat cardiomyocytes were assessed using a video-based edge-detection system (Ionoptix HyperSwitch Myocyte System). Cardiomyocytes were cultured on coverslips ($0.3\times10^6$ cells/coverslip) and rendered quiescent. Following treatments, coverslips were placed on a chamber mounted on the stage of an inverted microscope and perfused with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 10 glucose and 10 HEPES at pH7.4 and maintained at 37°C. Cells were stimulated to contract using the IonOptix Myopacer at a frequency of 0.5 Hz. Cardiomyocytes were displayed on a monitor display using an IonOptix Myocam camera. SoftEdge software (IonOptix) was used to compare changes in cell length during shortening (contraction) and relengthening (relaxation). Indices used to evaluate cell contractility included maximal velocity of shortening (+dL/dt) and maximal velocity of relengthening (-dL/dt). These are representations of systolic contraction and diastolic relaxation, respectively. Contractility was also measured as peak shortening.

## 3.12 *In vivo* animal study

The effects of resveratrol, pterostilbene and gnetol on cardiac structure and function were studied *in vivo* using the spontaneously hypertensive heart failure (SHHF) rat model with age-matched

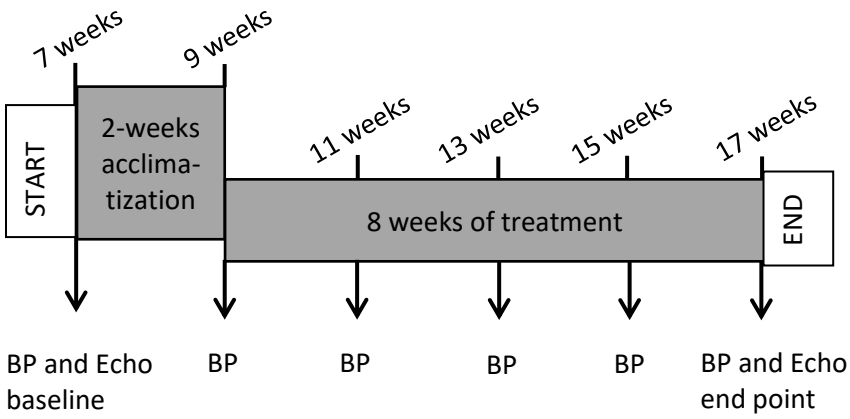
Sprague-Dawley (SD) rats serving as control. Experimental animals were ordered from Charles River (Senneville, Quebec, Canada). Thirty-two male SD rats and 32 male SHHF rats were obtained at 7 weeks of age. The animals were subjected to a 2-week period of acclimatization in the animal holding facility and exposed to 12-hour light and dark cycle at 22°C and 60% humidity.

## 3.13 Dosing and animal groups

All drugs were prepared at concentrations of 1 mg/ml in 50% ethanol vehicle. and daily doses were administered by oral gavage based on weekly weight measurements. The volumes of oral gavage administered varied from week to week and ranged from 600 µL to 1300 µL.

The SD and SHHF rats were divided into 4 groups of 8 animals each. Each group of 8 animals was treated with the vehicle (control), resveratrol, pterostilbene or gnetol. A dose of 2.5 mg/kg/day was chosen based on previous studies that showed prevention of cardiac hypertrophy[319] and vascular improvement[318] with 2.5 mg/kg/day of resveratrol in the spontaneously hypertensive rat (SHR) model. Animals were treated for a period of 8-weeks and fed *ad libitum* all through the study. Figure 7 represents a chart of the study timeline from start to finish.

**Figure 7: Treatments and study timeline**



## 3.14 Body Weight measurements

Body weights of were recorded each week and used to achieve correct dosing throughout the study.

## 3.15 Blood pressure measurements

After acclimatization, animals were trained for blood pressure measurements by tail cuff plethysmography (CODA non-invasive blood pressure system, Kent Scientific, Torrington, CT). Baseline blood pressure was taken at the beginning of the study and every 2 weeks until the end of the study.

## 3.16 Echocardiography

In collaboration with Dr. T. Netticadan and Pema Raj, transthoracic echocardiography was performed by 2D guided M-mode and Doppler imaging modalities with a 13-MHz probe (Vivid E9; GE Medical Systems, Milwaukee, WI) at the beginning and at the end of the treatment period. Rats were anaesthetized with 3% isoflurane in a chamber and images were obtained from rats under light anesthesia with 1.5 - 2% isoflurane, lying on their left side with the transducer placed on the left hemi-thorax outside the chamber on a heating pad. Two-dimensional parasternal long- and short-axis images of the left ventricle were obtained, and 2D targeted M-mode tracings were recorded from the parasternal short-axis view at the level of the papillary muscles. Doppler flow velocities tracing were obtained at the level of the mitral valve in the apical four-chamber view and at the level of the aorta in the five-chamber view with the Doppler probe placed at the edge of the mitral leaflets and aortic valve, respectively.

All measurements were performed according to the recommendations of the American Society for Echocardiography leading-edge method from three consecutive cardiac cycles using EchoPAC

software (GE Medical Systems, Milwaukee, WI). Systolic parameters such as percentage of left ventricular ejection fraction (EF) and fractional shortening were measured from the parasternal short-axis view image and based on end-systolic and end-diastolic diameters and volumes. Diastolic parameters such as IVRT were obtained from Doppler tracing. Structural parameters such as interventricular septal wall thickness (IVS), LV posterior wall thickness (LVPW) at diastole and LV internal dimension (LVID) at diastole and systole and relative wall thickness (RWT) were determined from parasternal short-axis view images.[319]

## 3.17 Lysate preparation

Cells: after treatment periods, cells were washed with ice cold PBS (containing protease inhibitors) and adherent cells were harvested by scraping from the plate wells and suspended in ~40 µl of freshly prepared RIPA buffer (containing protease inhibitors). The scraped cells were then incubated for 15-20 min on ice to allow for complete cell lysis and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was removed and used in the western blotting analysis. All steps were carried out on ice to prevent protein degradation.

Tissue: at the *in vivo* study endpoint (17 weeks of age), rats were anesthetized by 3% isoflurane and hearts were excised. The heart was washed in ice cold PBS to remove excess blood, weighed and stored frozen in storage buffer (RNA later containing 466.7 g/L ammonium sulphate, 16.7 µM sodium citrate, 13.3 µM EDTA, pH 5.2) at -80°C. To prepare the lysates, about 3 mL/g of freshly prepared RIPA buffer (containing protease inhibitors) was used to homogenize left ventricular tissue. After homogenization (Polytron homogenizer), the lysate was clarified by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was removed and normally diluted 1 in 6 in RIPA buffer before proceeding to western blot analysis.

## 3.18 Western blotting

Cell and tissue lysates were prepared in freshly prepared RIPA buffer containing protease and phosphatase inhibitors. Proteins of interest were detected by conventional western blotting under optimized conditions (Table 2). PVDF membranes were stripped and re-probed with β-actin antibody as the loading control.

**Table 2: Antibody list: sources, dilutions and incubation conditions**

| Antibody | Source (catalogue #) | Dilution, incubation |
|---|---|---|
| **AMPK** | Cell Signaling (2603S) | 1 in 2,000, overnight, 4°C |
| **p-AMPK** | Cell Signaling (2535S) | 1 in 2,000, overnight, 4°C |
| **AMPK$_{\alpha 1}$** | Millipore (92590) | 1 in 1,000, overnight, 4°C |
| **AMPK$_{\alpha 2}$** | Santa Cruz (sc-19131) | 1 in 600, overnight, 4°C |
| **β-actin** | Sigma (A5441) | 1 in 25-50,000 1 hour, RT |
| **MITF** | Abcam (Ab12039) | 1 in 4,000, overnight, 4°C |
| **Collagen I** | Cedarlane (CL50141AP-1) | 1 in 8,000, overnight, 4°C |
| **alpha-SMA** | Sigma (A2547) | 1 in 4,000, 1 hour, RT |
| ***Anti-alpha-actinin** | Sigma (A7811) | 1 in 700, overnight, 4°C |

**for immunofluorescent staining*

## 3.19 Statistical analysis

All data presented are mean values ± standard deviation (n=3-8). Statistical analyses were performed using GraphPad Prism version 6. One-way ANOVA followed by Neuman-Keuls multiple comparison test was used to detect differences between groups. A p value of $<0.05$ was considered statistically significant.

## 3.20 Animal ethics

Experimental animal protocols for this study were approved by the Animal Care Committee of the University of Manitoba and follow Canadian Council of Animal Care guidelines.

# CHAPTER 4

# ANTIHYPERTROPHIC EFFECTS OF STILBENES

# 4.0 CHAPTER FOUR: ANTIHYPERTROPHIC EFFECTS OF STILBENES

## 4.1 Effects of resveratrol on ET-1-induced hypertrophy

ET-1 (0.1 µM) was used to induce hypertrophy in neonatal rat cardiomyocytes. Although the anti-hypertrophic effect of resveratrol has been widely reported, [24, 26, 455] the effect of resveratrol on ET-1-induced hypertrophy was tested in cultured neonatal rat cardiomyocytes to verify that resveratrol is active in our model and cell type. The effect of increasing concentrations of resveratrol on ET-1-induced hypertrophy was determined. Figure 8 shows that 1, 5, and 10 µg/mL of resveratrol inhibited ET-1 induced hypertrophy.

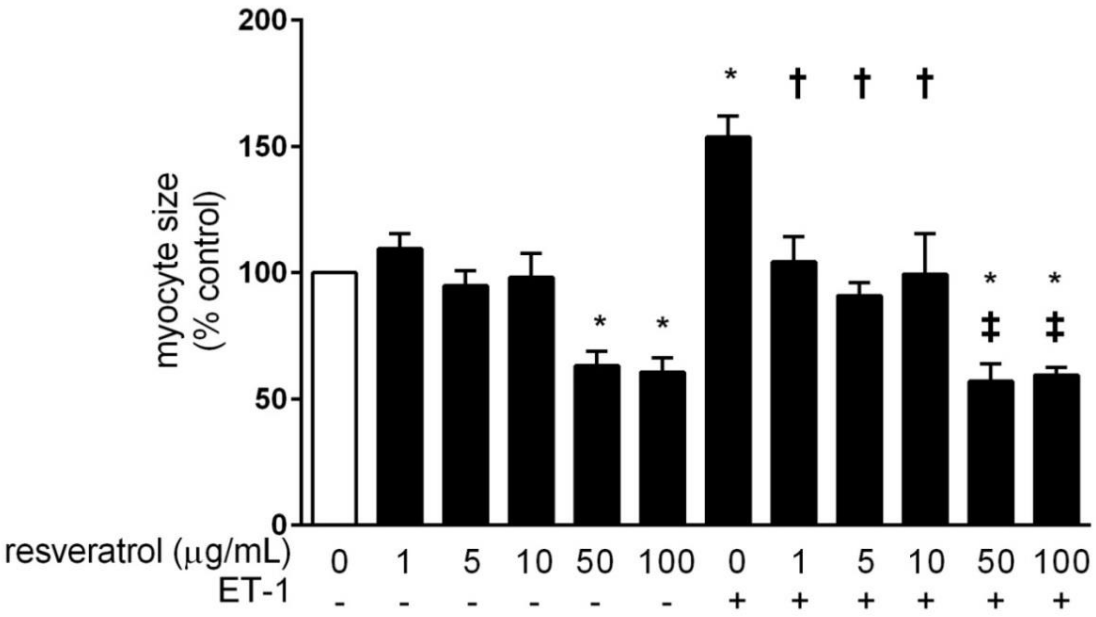



**Figure 8: Effect of resveratrol on ET-1 induced hypertrophy**

ET-1 treatment (0.1 µM, 24 h) increased cardiomyocyte size. 1 h pre-treatment with resveratrol at concentrations 1, 5, and 10 µg/mL prevented myocyte enlargement. Higher resveratrol concentrations (50 and 100 µg/mL) elicited significant reduction in cell size and changes in cell morphology in both control and ET-1-treated cells (n=3; *p<0.05 vs. control; †p<0.05 and ‡p<0.01 vs. ET-1).[466]

## 4.2 Effects of gnetol on cardiomyocyte viability

To identify concentrations of gnetol that are non-toxic to cardiomyocytes for further experiments, a viability assay was carried out using calcein AM staining on the cells and 0.2% Triton X-100 as the dead control. Figure 9 shows that gnetol concentrations of 1, 5, and 10 µg/mL did not significantly reduce cell viability, measured as calcein fluorescence, while concentrations 50 and 100 µg/mL resulted in a significant reduction in cell viability. Thus, a concentration of 5 µg/mL gnetol was chosen for the rest of the experiments.

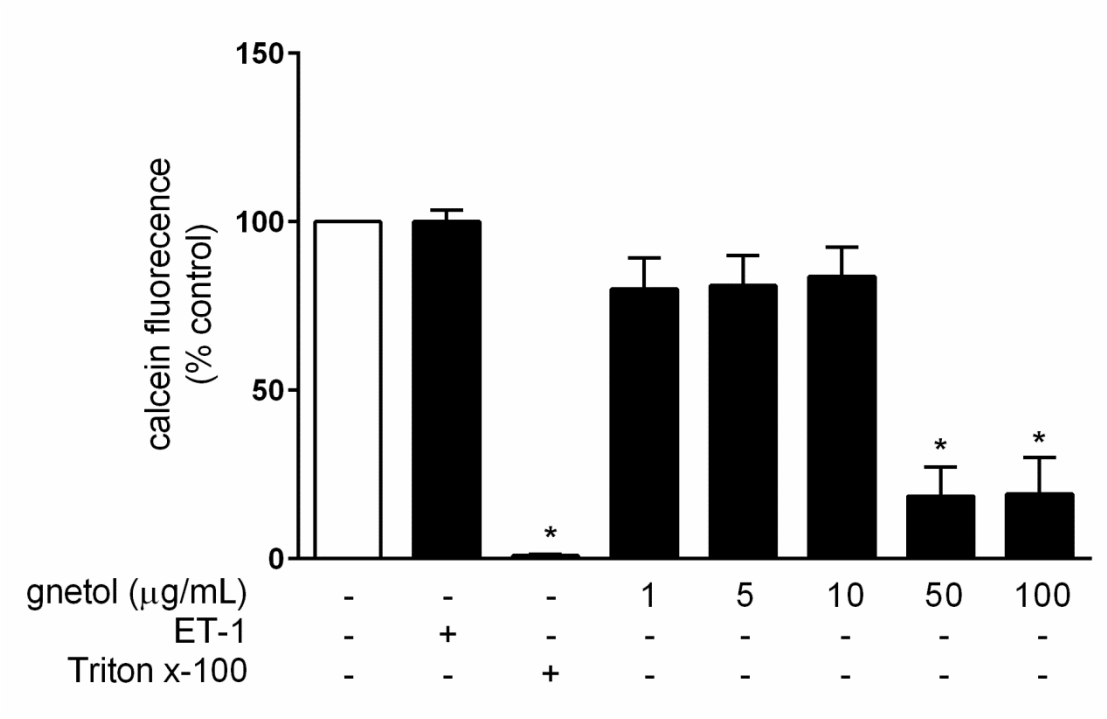



**Figure 9: Effect of gnetol on cardiomyocyte viability**

Cardiomyocytes were treated with gnetol at concentrations 1, 5, 10, 50 and 100 µg/mL. After 24 h, viability was assessed by staining with the fluorescent dye, calcein AM. Cardiomyocytes treated with 0.2% Triton X-100 showed minimal fluorescence compared to the control. ET-1 (0.1 µM) and gnetol (1, 5, and 10 µg/mL) did not significantly affect cardiomyocyte viability; however, 50 and 100 µg/mL of gnetol reduced viability (n=3-4; *p<0.05 vs. control).[466]

## 4.3 Gnetol inhibits hypertrophy in neonatal rat cardiomyocytes

Figure 10 shows that gnetol prevented the increase in cell size, protein synthesis (AHA incorporation) and fetal gene expression induced by ET-1. Different concentrations of gnetol were used in the initial cell size experiment, and gnetol was effective at 1, 5, and 10 µg/mL in preventing cell growth. However, at much higher concentrations of 50 and 100 µg/mL, gnetol caused significant reduction of cardiomyocyte size.

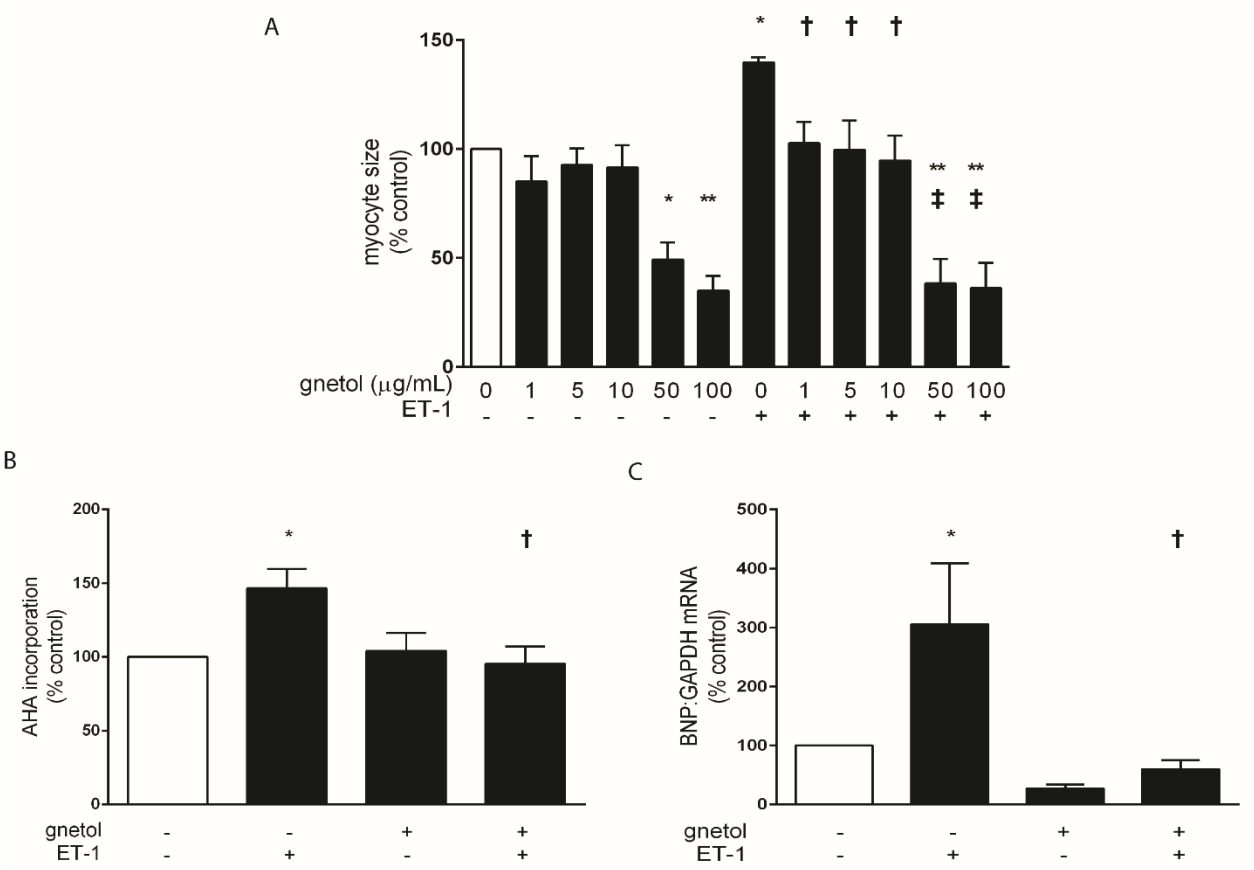



**Figure 10: Effect of gnetol on the three markers of hypertrophy**

A) Gnetol (1, 5, 10 µg/mL) inhibited ET-1-induced myocyte size growth (n=3; *p<0.05 and **p<0.01 vs. control; †p<0.05 and ‡p<0.01 vs. ET-1). Gnetol (5 µg/mL) also inhibited B) protein synthesis (n=3; *p<0.05 vs. control; †p<0.05 vs. ET-1),[466] and C) expression of BNP (n=3-5; *p<0.05 vs. control; †p<0.05 vs. ET-1) stimulated by ET-1 treatment in neonatal rat cardiomyocytes.

## 4.4 Effects of gnetol on adult cardiomyocyte contractility

Here, the effect of gnetol on parameters of adult cardiomyocyte contractility such as maximum shortening velocity, maximum re-lengthening velocity and fractional shortening, was probed. ET-1 treatment caused a decline in all three parameters (Figure 11). Although gnetol was unable to rescue the decline in contractility induced by ET-1, it is important to note that gnetol treatment did not significantly impair contractility.

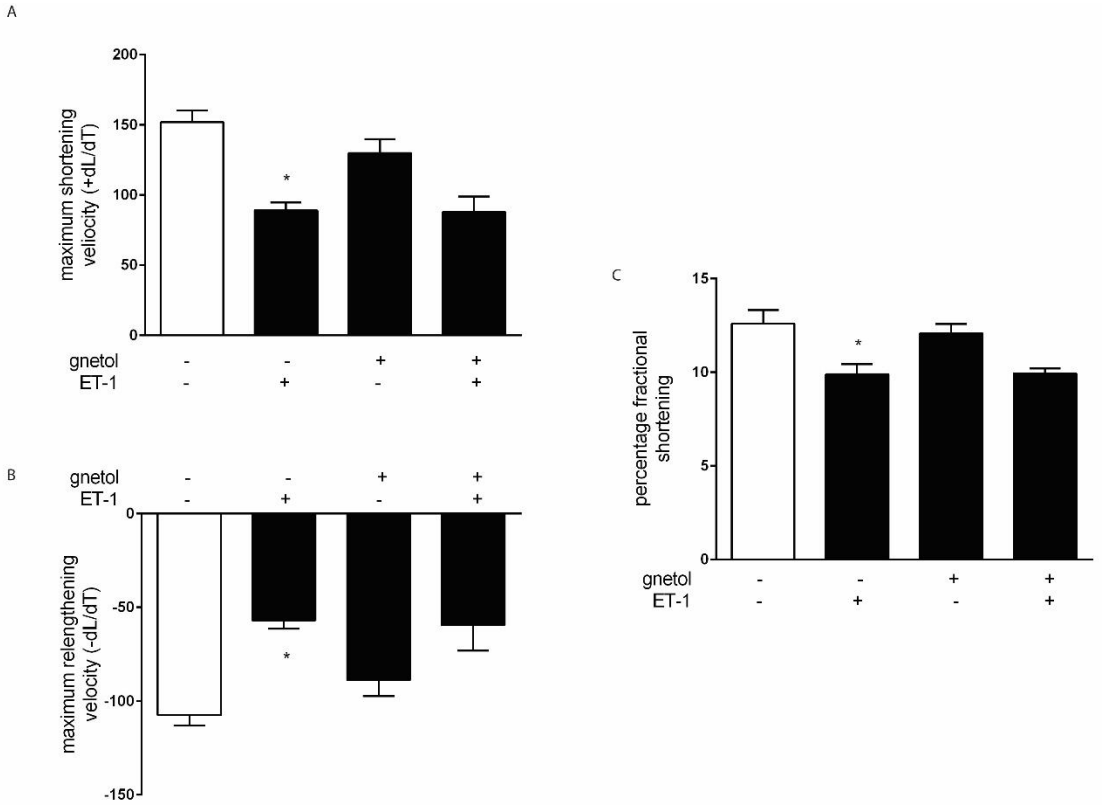



**Figure 11: Effect of gnetol on adult cardiomyocyte contractility**

ET-1 treatment reduced A) maximum shortening velocity, B) maximum re-lengthening velocity and C) percentage fractional shortening. Gnetol (5 µg/mL) neither improved nor worsened these parameters (n=5-9; *p<0.05 vs. control).

## 4.5 Effects of pterostilbene on cardiomyocyte viability

To identify concentrations of pterostilbene that are non-toxic to cardiomyocytes for further experiments, a viability assay was conducted using calcein AM staining and 0.2% Triton X-100 as the dead control. Figure 12 shows that pterostilbene concentrations of 5, 10 and 50 µg/mL significantly reduced cell viability. However, 1 µg/mL did not impair calcein fluorescence. Thus, 1 µg/mL pterostilbene was chosen for the rest of the experiments.

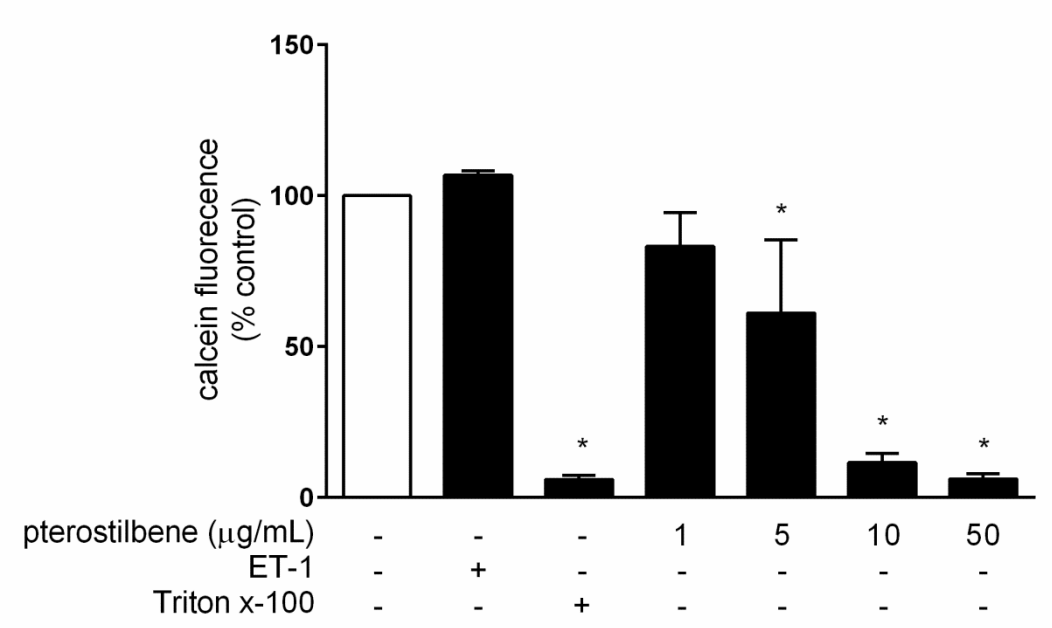



**Figure 12: Effect of pterostilbene on cardiomyocyte viability**

Cardiomyocytes were treated with pterostilbene at concentrations of 1, 5, 10 and 50 µg/mL. After 24 h, viability was assessed by staining with the fluorescent dye, calcein AM. Cardiomyocytes treated with 0.2% Triton X-100 showed minimal fluorescence compared to the control. ET-1 (0.1 µM) and pterostilbene (1 µg/mL) did not significantly affect cardiomyocyte viability; however, 5, 10 and 50 µg/mL of pterostilbene reduced cell viability (n=3-4; *p<0.05 vs. control).[466]

## 4.6 Pterostilbene inhibits hypertrophy in neonatal rat cardiomyocytes

Similar to gnetol, the effect of pterostilbene (1 µg/mL) was evaluated on three markers of hypertrophy: cell size, protein synthesis and fetal gene (BNP) expression (Figure 13). Pterostilbene prevented increases in myocyte size and protein synthesis when treated with ET-1. However, pterostilbene did not prevent BNP expression induced by ET-1. BNP mRNA levels remained high in cardiomyocytes treated with pterostilbene and ET-1.

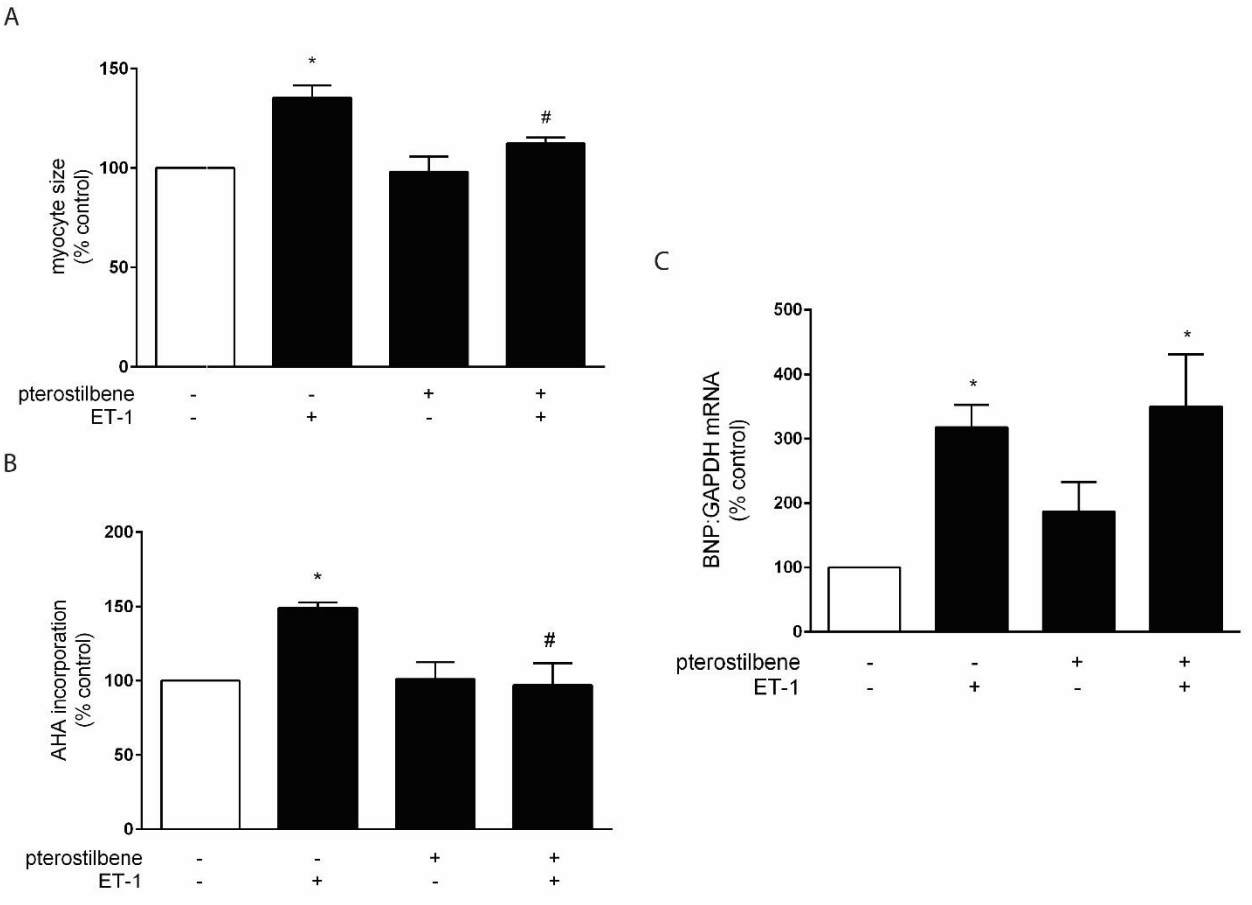



**Figure 13: Effects of pterostilbene on three markers of hypertrophy**

A) Pterostilbene (1 µg/mL) prevented the increase in cell size induced by ET-1 (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1); B) pterostilbene (1 µg/mL) also prevented the increase in protein synthesis caused by ET-1 (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1).[466] However, C) pterostilbene (1 µg/mL) failed to prevent BNP induction (n=3; *p<0.05 vs. control).

# CHAPTER 5

# ANTIHYPERTROPHIC SIGNALING MECHANISMS

# 5.0 CHAPTER FIVE: ANTIHYPERTROPHIC SIGNALING MECHANISMS

## 5.1 Gnetol activates AMPK via phosphorylation

In a time-course experiment, the effects of gnetol on phosphorylated AMPK (p-AMPK), which is the activated form of the protein, and total AMPK (t-AMPK) were determined using the western blotting technique. Figure 14A shows that gnetol did not significantly increase t-AMPK at time points 4, 8, or 24 h. In contrast, gnetol increased p-AMPK levels significantly (~2.5 fold) at 4 h (Figure 14B). This implies that gnetol activated AMPK transiently at the 4-hour time point, while p-AMPK level returned to basal levels after 24 h.

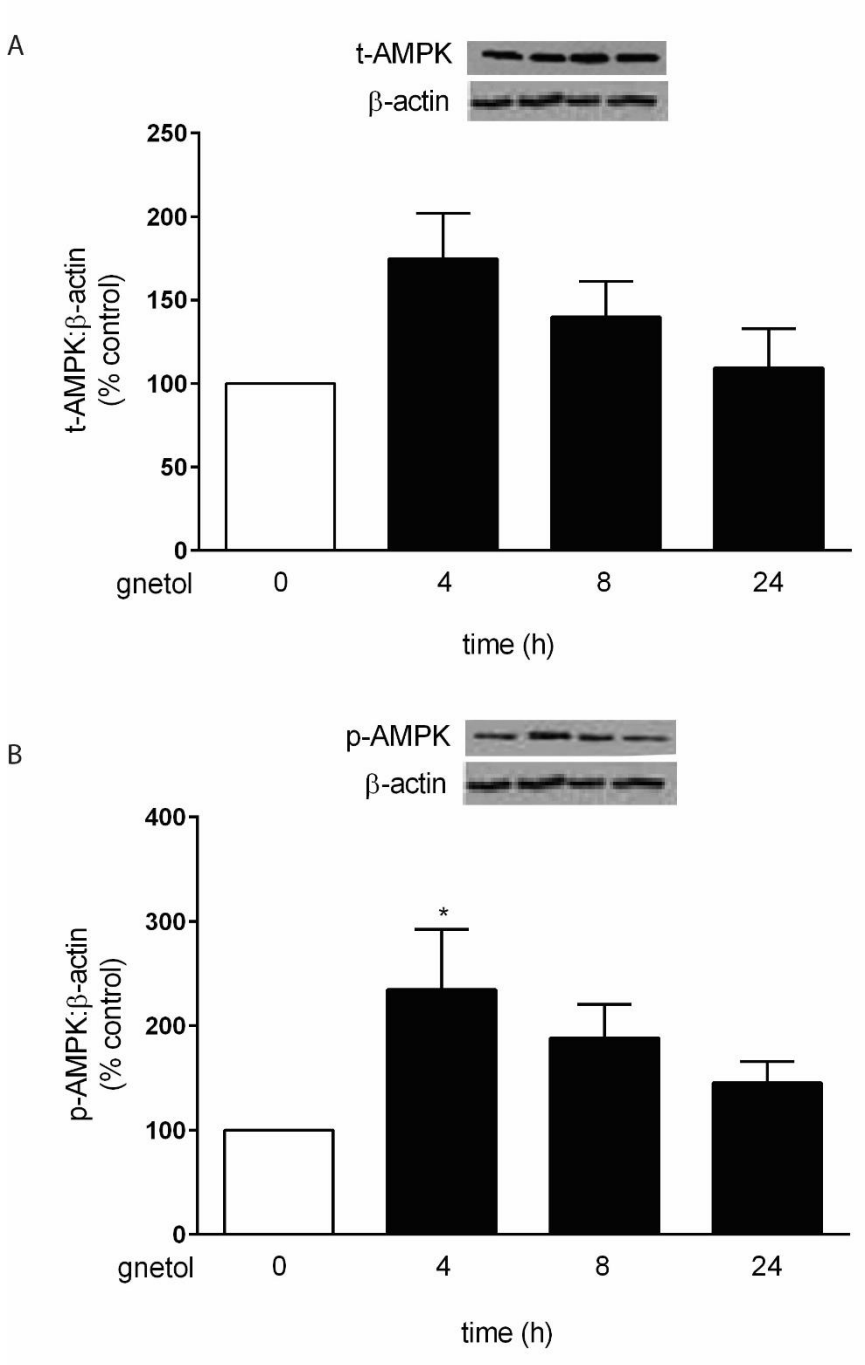



Figure 14: Effect of gnetol on AMPK activation (total AMPK and phosphorylated AMPK)

Cardiomyocytes were treated with gnetol (5 µg/mL) in a time-course experiment. After specific time points, cardiomyocytes were harvested, protein extracted and blotted for t-AMPK and p-AMPK. A) Gnetol did not significantly increase levels of t-AMPK; (n=3-5). B) Gnetol significantly increased p-AMPK at 4 h; (n=3-5; *$p<0.05$ vs. 0 h).[466]

## 5.2 Inhibition of AMPK abolishes the anti-hypertrophic effects of gnetol

In order to implicate AMPK in the anti-hypertrophic effects of gnetol, cardiomyocytes were pretreated with a pharmacological inhibitor of AMPK compound C, and the cell size experiments repeated. Compound C, also known as dorsomorphin, is a cell-permeable competitive inhibitor of AMPK activity. Figure 15 shows that in the presence of compound C, gnetol was unable to inhibit cardiomyocyte hypertrophy.

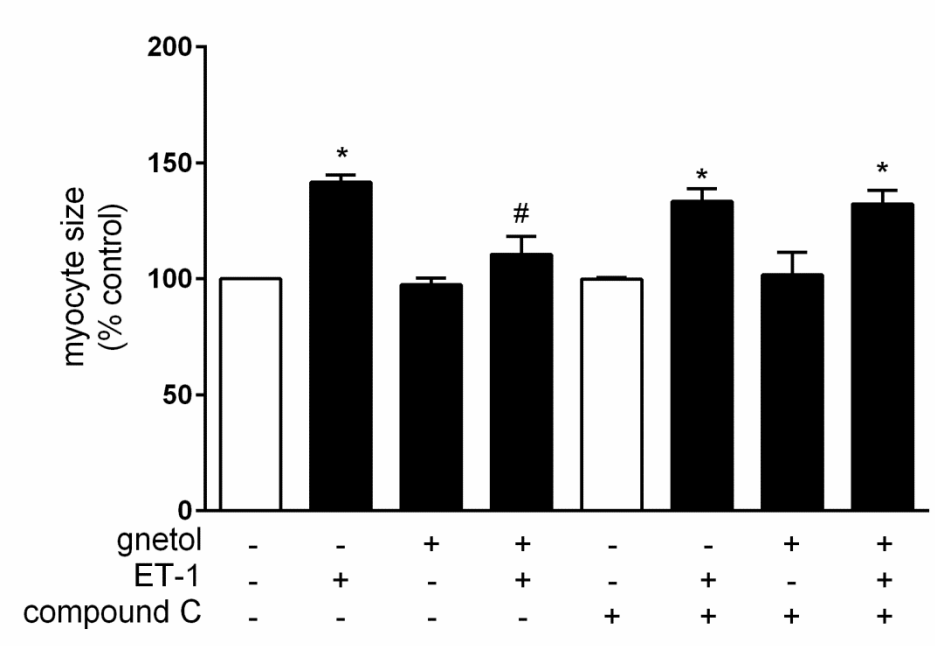



**Figure 15: Effect of compound C, a pharmacological inhibitor of AMPK, on anti-hypertrophic effects of gnetol**

In the presence of compound C, (AMPK inhibitor; [1 μM]), the ability of gnetol (5 μg/mL) to inhibit cardiomyocyte growth was abolished (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1).[466]

## 5.3 Genetic knockdown of AMPK abolishes the anti-hypertrophic effects of gnetol

To further confirm the role of AMPK activation as the signaling mechanism involved in the anti-hypertrophic actions of gnetol, cardiomyocytes were infected with lentiviral constructs expressing shRNA against AMPKα1/2 isoforms and the cell size experiments were repeated. Figure 16 shows that similar to the effects of compound C, gnetol also lost the ability to reduce cardiomyocyte size in the presence of AMPKα1/2 shRNA.

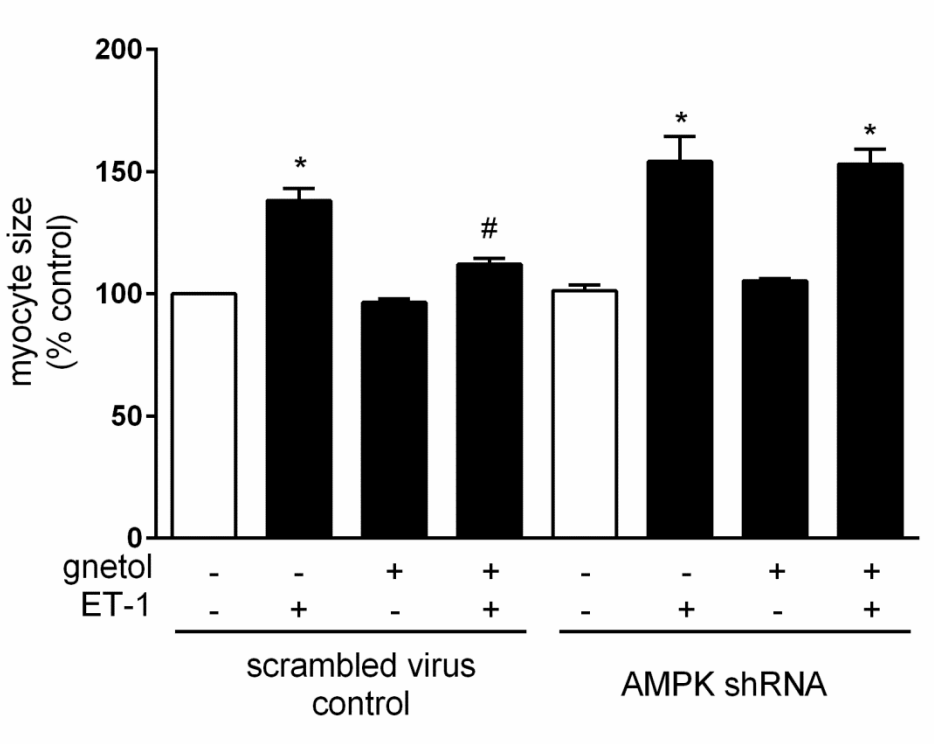



Figure 16: Effect of shRNA silencing of AMPK on the anti-hypertrophic effect of gnetol

Whereas in the presence of scrambled virus control, the anti-hypertrophic effect of gnetol (5 µg/mL) was preserved, AMPKα knockdown disrupted the ability of gnetol to prevent cardiomyocyte growth. (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1).[466]

## 5.4 Pterostilbene activates AMPK via phosphorylation

The effect of pterostilbene on AMPK activation was probed. Figure 17A shows that pterostilbene increased t-AMPK expression at time points 8 and 24 h. Pterostilbene also increased p-AMPK at time points 4, 8 and 24 h (Figure 17B). This indicates that pterostilbene activated AMPK in a sustained fashion from 8 h to 24 h.

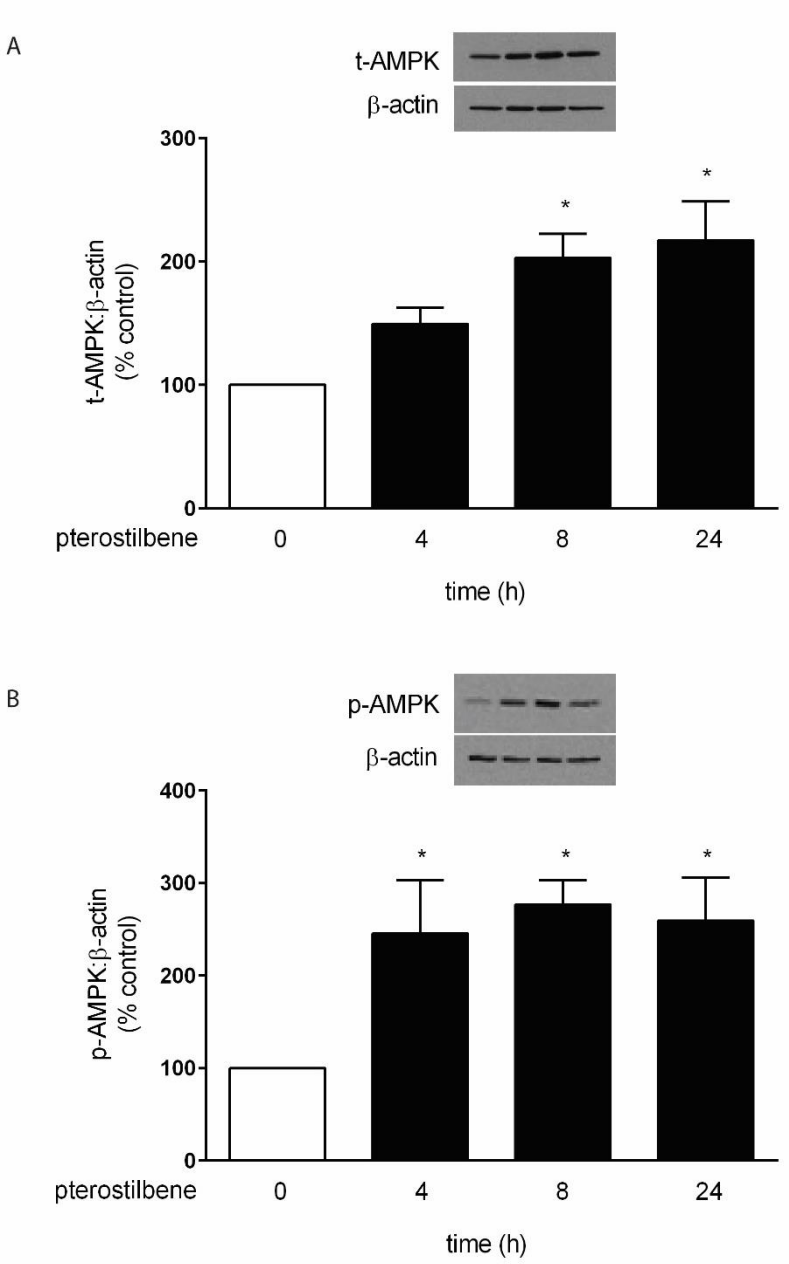



Figure 17: Effect of pterostilbene on AMPK activation (total AMPK and phosphorylated AMPK)

Cardiomyocytes were treated with pterostilbene (1 µg/mL) in a time-course experiment. After specific time points, cardiomyocytes were harvested, protein extracted and blotted for t-AMPK and p-AMPK. Pterostilbene significantly increased A) t-AMPK levels at time points 8 and 24 h, and B) p-AMPK at time points 4, 8 and 24 h (n=3, *$p<0.05$ vs. 0 h).[466]

## 5.5 Inhibition of AMPK abolishes anti-hypertrophic effects of pterostilbene

To explore the role of AMPK in the anti-hypertrophic effects of pterostilbene, cardiomyocytes were pretreated with compound C and the cell size experiments repeated. Figure 18 shows that in the presence of compound C, pterostilbene lost its anti-hypertrophic effects. Thus, it may be inferred that gnetol and pterostilbene inhibited ET-1-induced hypertrophy at least in part via activation of AMPK.

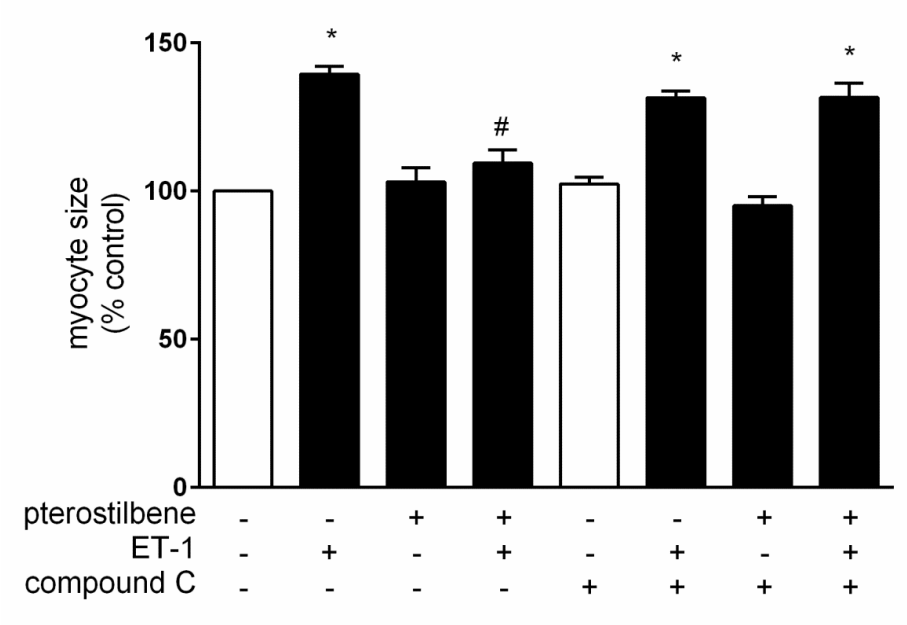



**Figure 18: Effect of compound C, a pharmacological inhibitor of AMPK on anti-hypertrophic effect of pterostilbene**

In the presence of compound C (AMPK inhibitor; [1 μM]), the ability of pterostilbene (1 μg/mL) to inhibit cardiomyocyte growth was abolished (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1).[466]

## 5.6 Genetic knockdown of AMPK abolishes the anti-hypertrophic effects of pterostilbene

To further implicate AMPK in the signaling mechanism of pterostilbene, cardiomyocytes were infected with lentiviral constructs expressing shRNA against AMPKα1/2 isoforms and the cell size experiments were repeated. Figure 19 shows that similar to the effects of compound C, pterostilbene also lost the ability to reduce cardiomyocyte size in the presence of AMPKα1/2 shRNA.

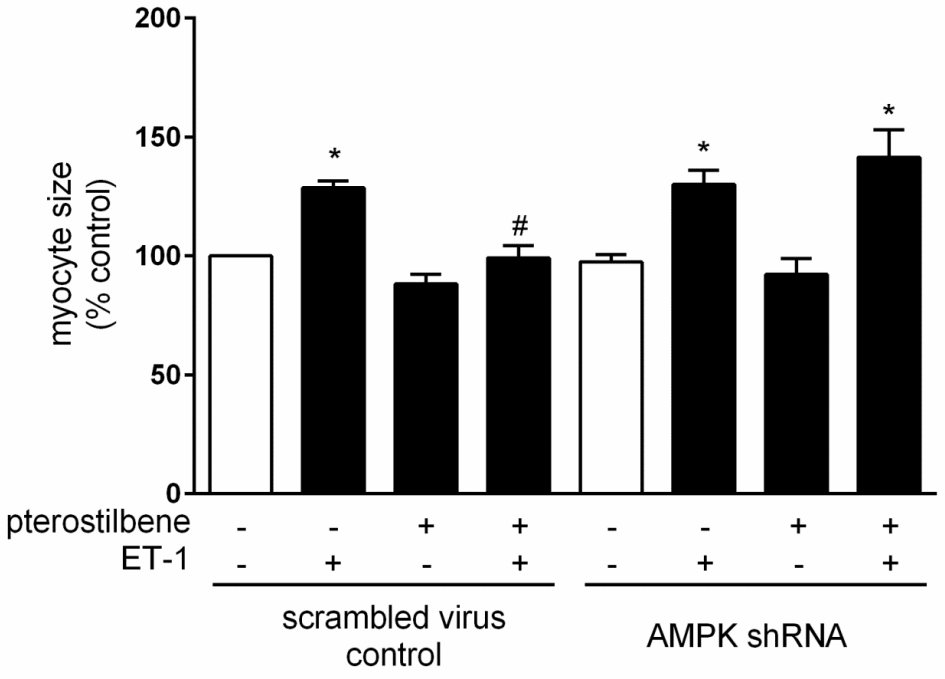



**Figure 19: Effect of shRNA silencing on anti-hypertrophic effect of pterostilbene**

Whereas, in the presence of scrambled virus control, the anti-hypertrophic effect of pterostilbene (1 µg/mL) was preserved, AMPKα knockdown disrupted the ability of pterostilbene to prevent cardiomyocyte growth; (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1).[466]

## 5.6 AMPK knockdown efficiency

Figure 20 shows the efficiency of shRNA silencing of AMPKα isoforms in this study as determined by western blotting. Protein levels of each AMPKα isoform (AMPK α1 and AMPK α2) and total AMPK were probed 6 days after infecting the cells with AMPKα1/2 shRNA. AMPKα1 protein level was reduced to 29.6%, AMPKα2 to 35.2% and total AMPK to 23.4% (Figure 20).

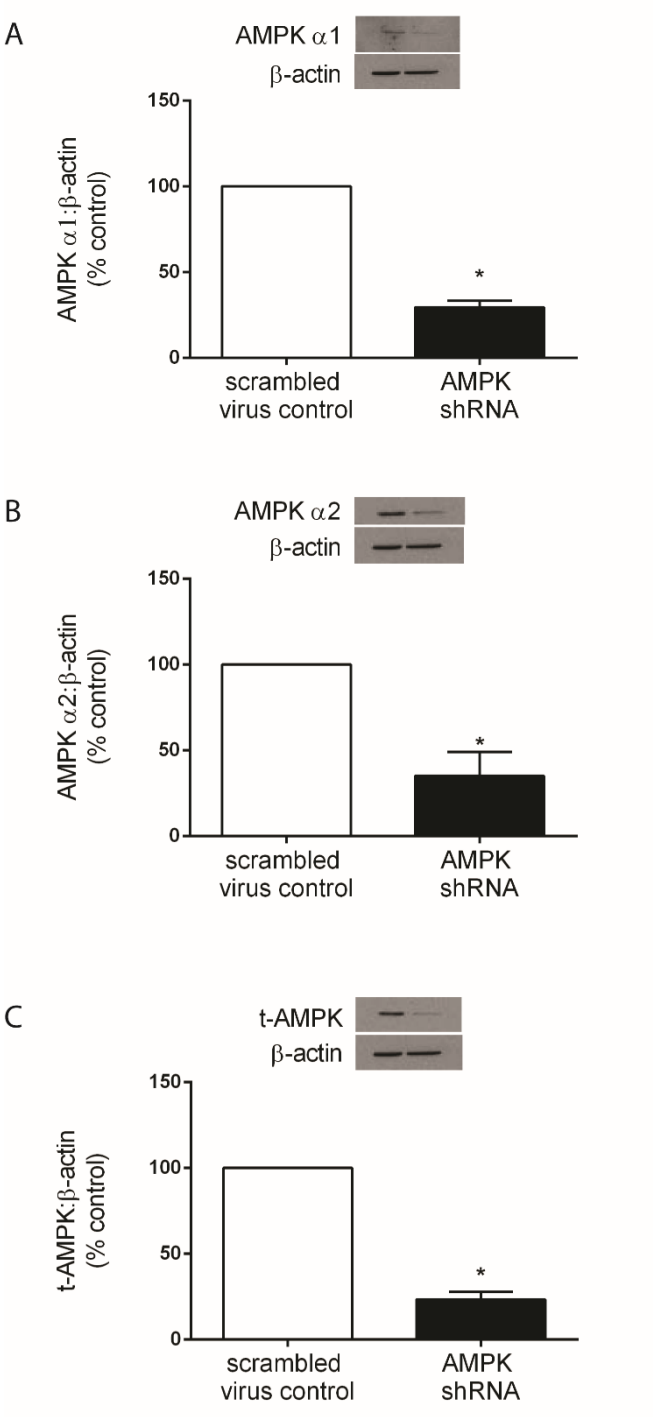



**Figure 20: shRNA silencing of AMPKα isoforms**

Expression of the A) AMPKα1 isoform, B) AMPKα2 isoform and C) total AMPK were significantly reduced after treatment with the AMPKα1/2 shRNA (n=3; *$p<0.05$ vs. scrambled virus control).

## 5.7 Pharmacological inhibition of MITF blunts hypertrophic response to ET-1

ML329 is a pharmacological inhibitor of MITF. ML329 alone had no significant effect on cardiomyocyte size. Compared to the ET-1 group which exhibited a 1.6-fold increase, ML329 + ET-1 treatment significantly reduced the size increase to only 1.3-fold. Despite reduction in myocyte size by ML329 + ET-1 treatment, the myocyte size was still significantly higher than the control group. Thus, pre-treatment with ML329 partially blunted ET-1-induced cardiomyocyte growth (Figure 21).

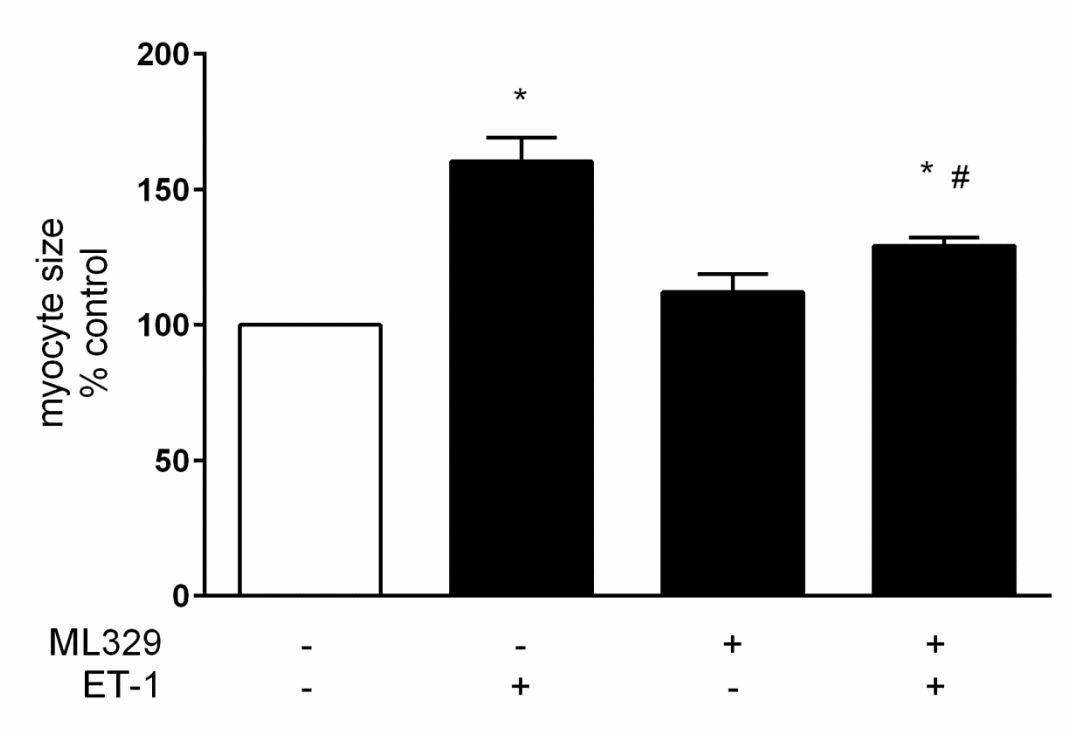



**Figure 21: Effect of the MITF inhibitor, ML329, on ET-1 induced hypertrophy**

Cardiomyocytes were treated with ET-1 (0.1 µM) after 1 hour pre-treatment with the MITF inhibitor, ML329 (2 µM). ML329 significantly reduced cardiomyocyte size compared to ET-1 treated group, but still significantly higher than the control group. (n=3; *p<0.05 vs. control; #p<0.05 vs. ET-1)

## 5.8 ET-1 increases MITF expression

ET-1 significantly increased MITF expression by about 2.5-fold at 30 min and 4 h compared to time 0 h (Figure 22). MITF expression returned to basal levels after 24 h.

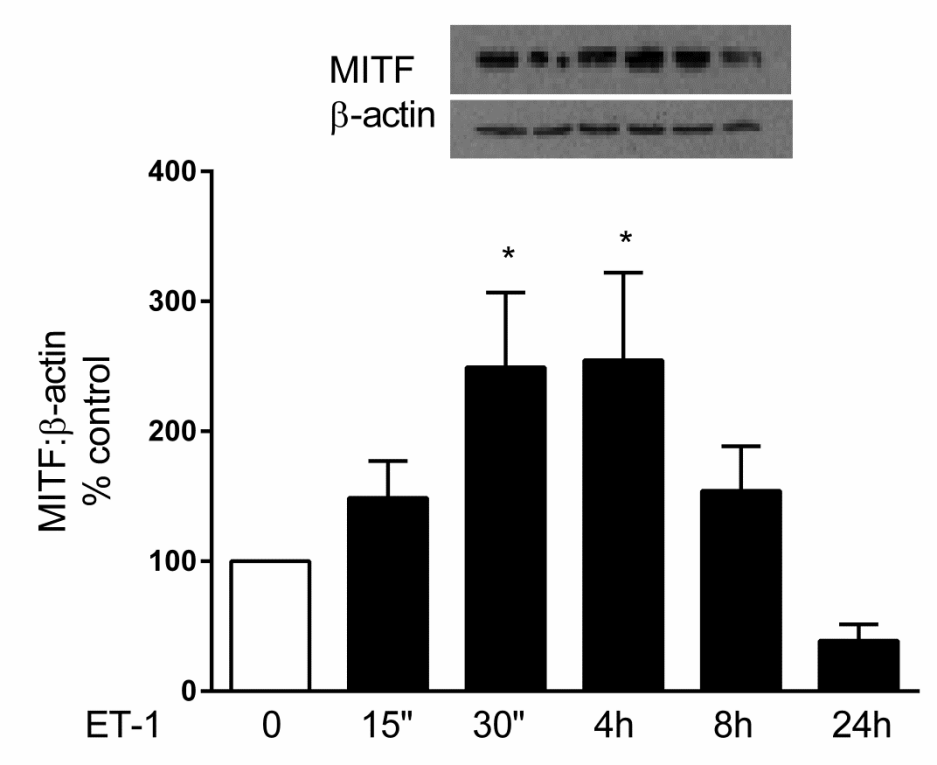



**Figure 22: Effect of ET-1 on MITF expression**

Time-course experiments show ET-1 increased MITF protein expression levels at 30 min and 4 h. After 24 h of treatment, MITF protein levels returned to basal levels. (n=4-6; *p<0.05 vs. 0 h)

## 5.9 Effects of stilbenes on MITF expression

The time-course effect of gnetol and pterostilbene on MITF expression was also examined. Cardiomyocytes were treated with either gnetol or pterostilbene. After specific time points, cardiomyocytes were harvested, protein harvested and blotted for MITF protein. Figure 23 shows that neither (A) gnetol nor (B) pterostilbene had significant effects on MITF expression over a period of 24 h.

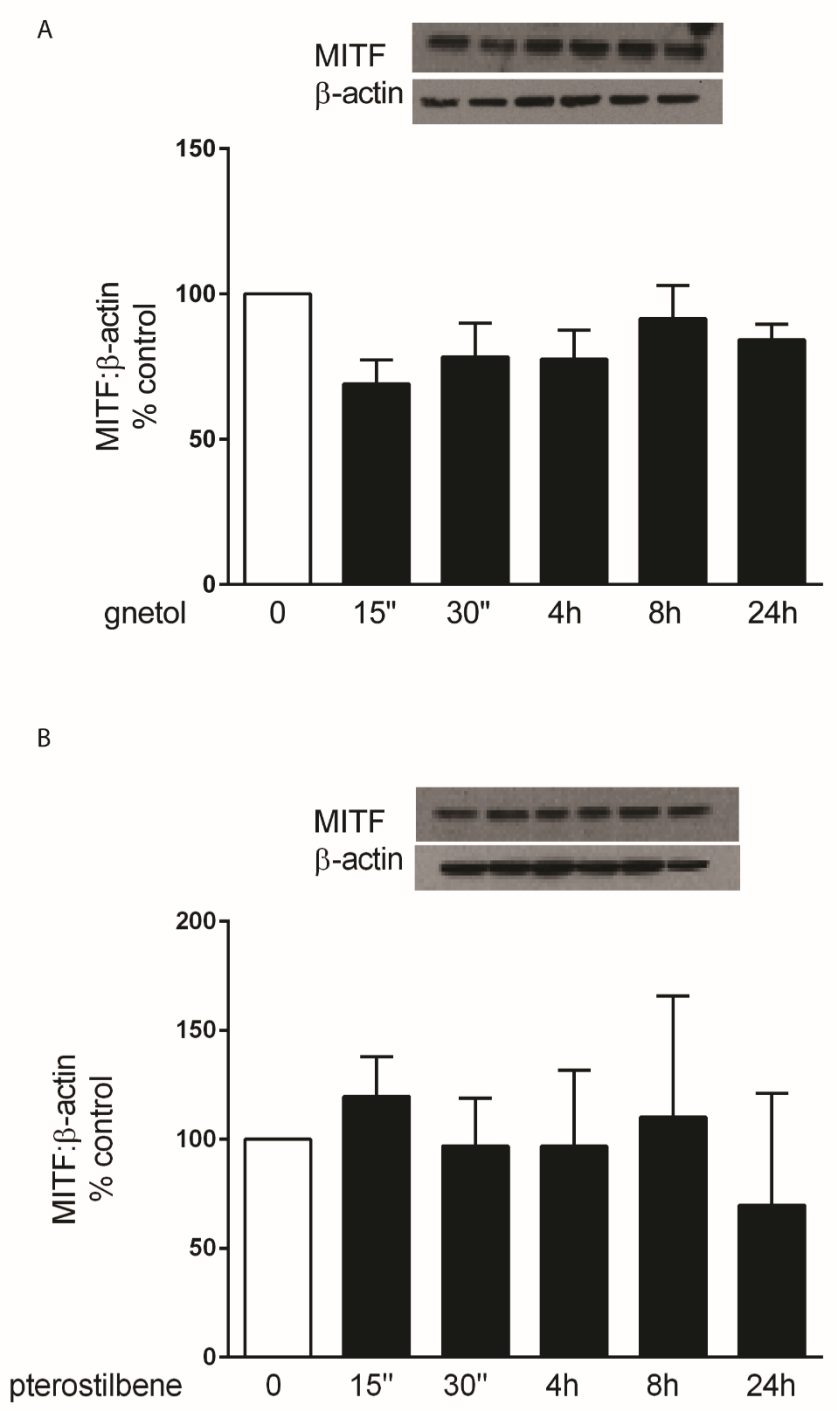



**Figure 23: Effects of gnetol and pterostilbene on MITF expression**

A) Gnetol (5 µg/mL) did not significantly change MITF protein levels over a period of 24 h (n=6-8). B) Similarly, pterostilbene (1 µg/mL) had no effect on MITF levels over a period of 24 h (n=3).

## 5.10 Stilbenoids prevented the increase in MITF expression induced by ET-1

Although gnetol or pterostilbene alone did not have significant effects on MITF expression, the effect of gnetol, pterostilbene and resveratrol pre-treatment on MITF expression induced by ET-1 was examined. ET-1 treatment resulted in a 1.7-fold increase in MITF expression (Figure 24). Here, a disparate effect of stilbenoids on MITF expression was observed. While gnetol and pterostilbene did not affect MITF expression, resveratrol increased MITF expression also by 1.7-fold. Of note, all stilbenoids including resveratrol prevented further increase in MITF expression in the ET-1 treated groups.

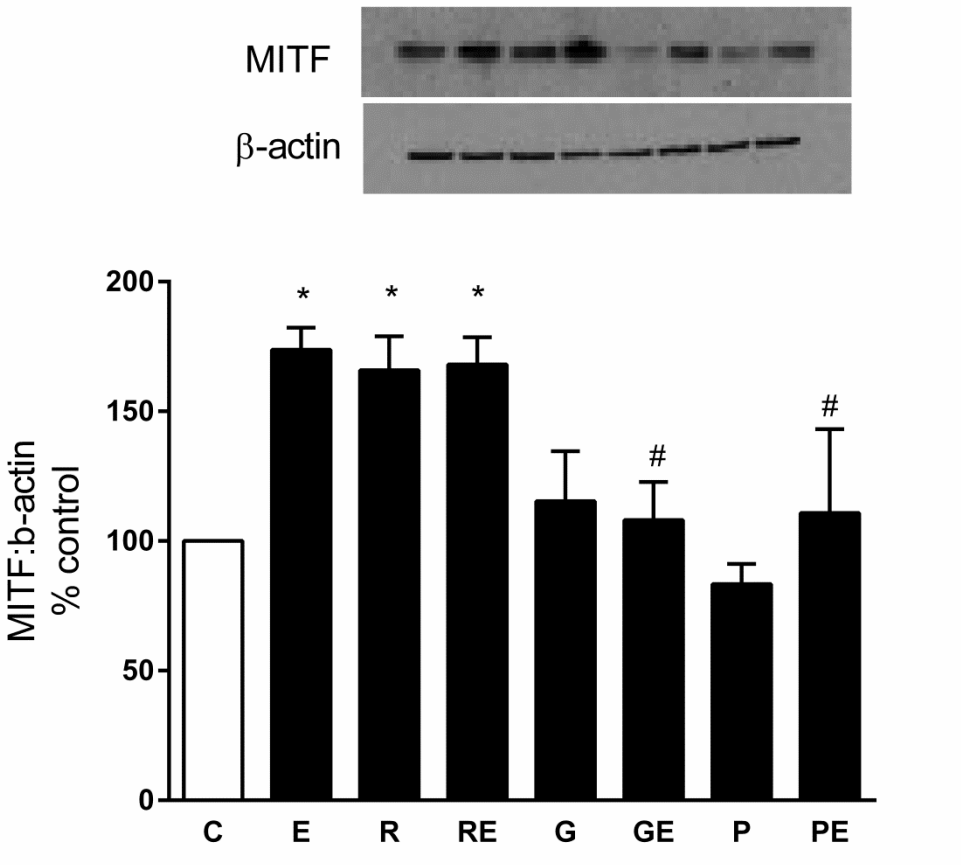



**Figure 24: Effects of stilbenoid pre-treatment on MITF expression with and without ET-1**

ET-1 increased MITF expression. Stilbenoids prevented further increase in MITF expression in the ET-1 treated groups. Interestingly, in contrast to gnetol and pterostilbene, which had no significant effect on MITF, resveratrol increased MITF expression (n=5-8; *p<0.05 vs. control; #p<0.05 vs. ET-1).

## 5.11 Pharmacological inhibition of AMPK blunts MITF expression

Since stilbenoids activated AMPK and also attenuated ET-1 –induced MITF expression, the notion of cross-talk between the AMPK and MITF pathways seemed interesting to pursue. Cardiomyocytes were pre-treated with compound C (1 µM; 1 h) followed by gnetol and pterostilbene in the presence and absence of ET-1 for 4 h. ET-1 did not increase MITF expression in the absence of compound C (Figure 25). Since there was no increase in MITF expression with ET-1 treatment, it is difficult to determine the effect stilbenoids on ET-1-induced changes in MITF in the presence of compound C.

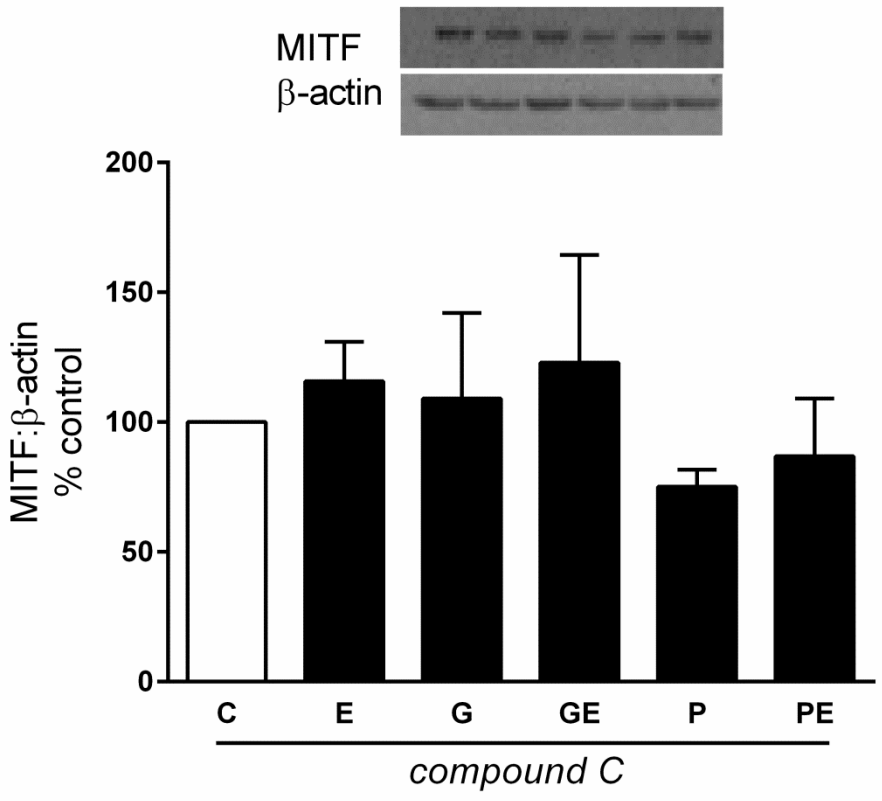



**Figure 25: Effect of compound C and stilbenoid pretreatment on MITF expression induced by ET-1**

In the presence of compound C [1 µM], ET-1 failed to stimulate increase in MITF expression. Cardiomyocytes were treated with gnetol (5 µg/mL) and pterostilbene (1 µg/mL) for 1 h followed by treatment with ET-1 for 4 h (n=3-4).

## 5.12 Effect of compound C on MITF expression with and without ET-1

To verify the effect of AMPK inhibition on MITF expression, the experiments were repeated using only compound C and ET-1. Here, ET-1 increased MITF expression by 1.7-fold but compound C prevented this increase (Figure 26). Compound C alone had no significant effect on MITF but prevented ET-1-induced MITF expression.

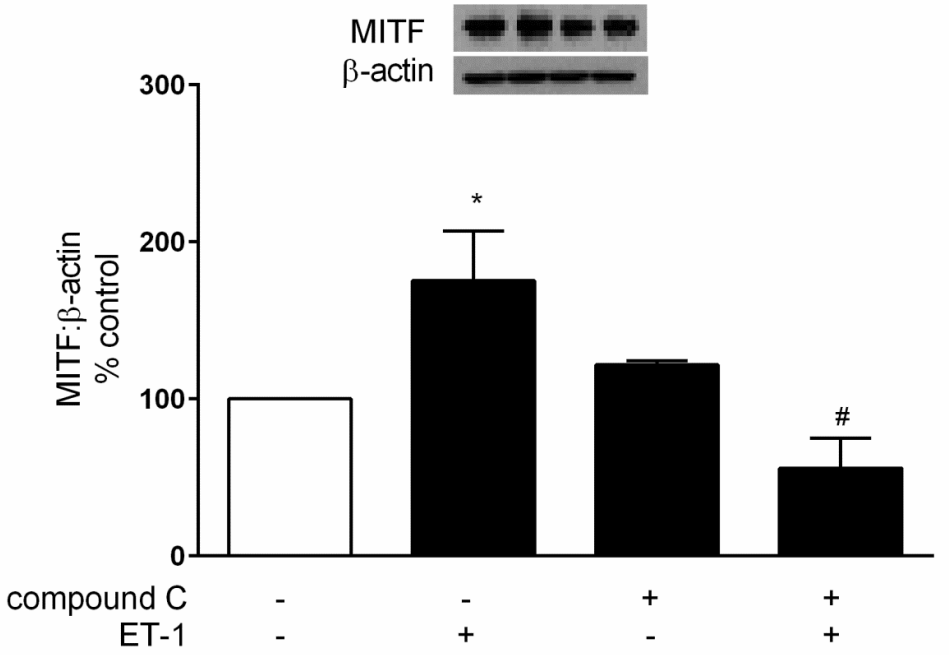



**Figure 26: Effect of compound C on ET-1 induced MITF expression**

Cardiomyocytes were treated with compound C [1 μM] for 1 h followed by ET-1 treatment for 4 h. ET-1 increased MITF expression. However, in the presence of compound C, ET-1 failed to induce MITF expression (n=3; *p<0.05 vs. control #p<0.05 vs. ET-1).

# CHAPTER 6

# EFFECTS OF STILBENOIDS ON CARDIAC STRUCTURE AND FUNCTION IN SPONTANEOUSLY HYPERTENSIVE HEART FAILURE RATS

# 6.0 CHAPTER SIX: EFFECTS OF STILBENOIDS ON CARDIAC STRUCTURE AND FUNCTION IN SPONTANEOUSLY HYPERTENSIVE HEART FAILURE RATS

## 6.1 Cardiac structure parameters

### 6.1.1 Left ventricular mass and body weights (normalized with tibia length)

The left ventricular masses and body weights were normalized with tibia length measured in centimetres (cm). There were no significant differences in normalized left ventricular mass (Figure 27A). Stilbenoid treatments had no effect on body weight over the course of the study. SHHF rats weighed significantly less than their SD counterparts. The normalized body weight of the untreated SHHF rats was 99.2 g/cm compared to 130.9 g/cm in the untreated SD rats at age 17 weeks (Figure 27B).

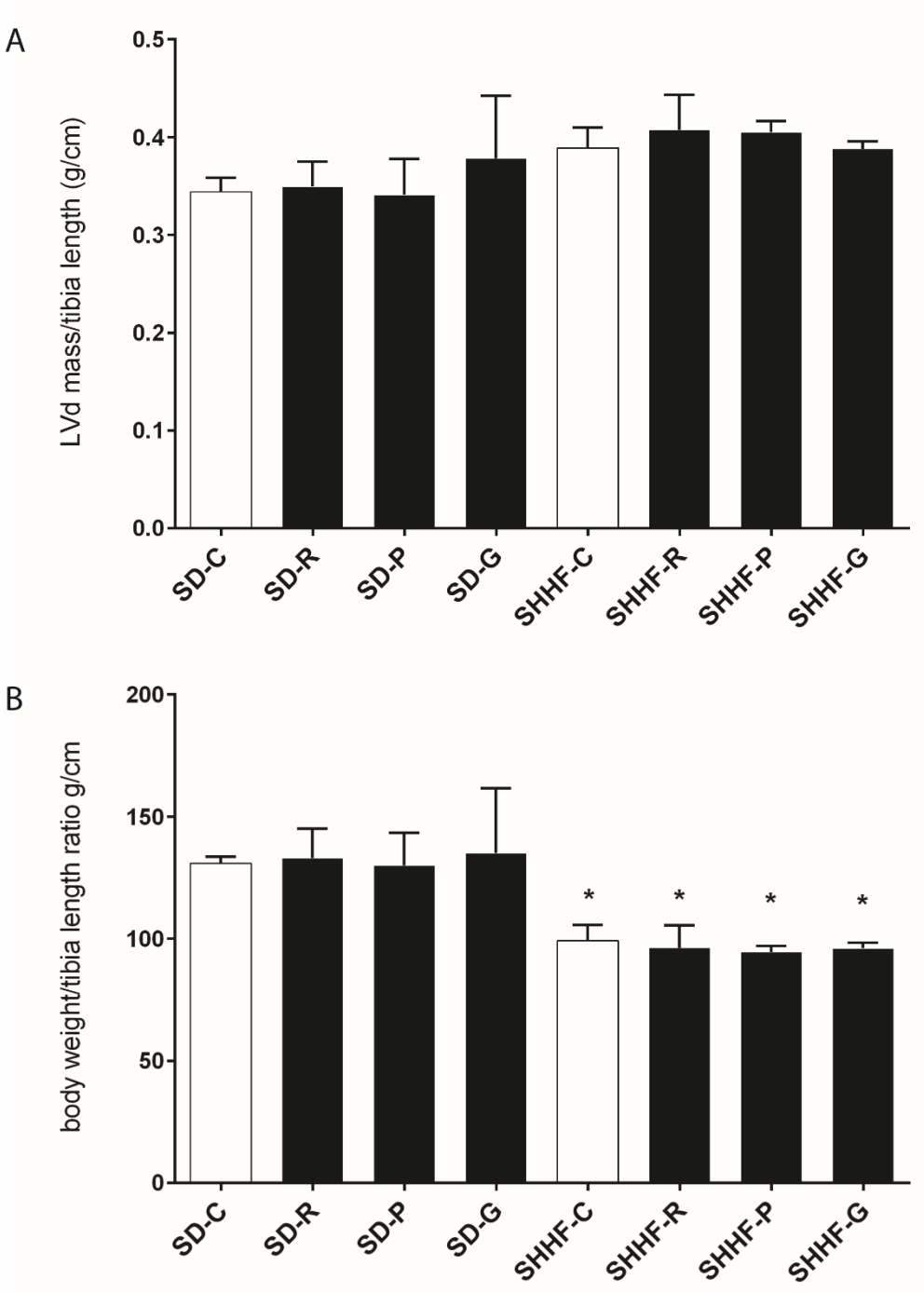



**Figure 27: Effects of stilbenoids on left ventricular mass and body weights**

A) Normalized left ventricular mass was not significantly increased in the untreated SHHF group (SHHF-C) compared to the SD-C group at age 17 weeks. Stilbenoids also did not have any significant effect on the left ventricular mass within the SHHF or SD groups. B) Normalized body weights of SHHF group were significantly less than that of the SD groups at age 17 weeks. Stilbenoids did not influence the body weights of animals both in the SD and in the SHHF rat group (n=6-8; *$p<0.05$ vs. SD-C).

### 6.1.2 Treatment groups

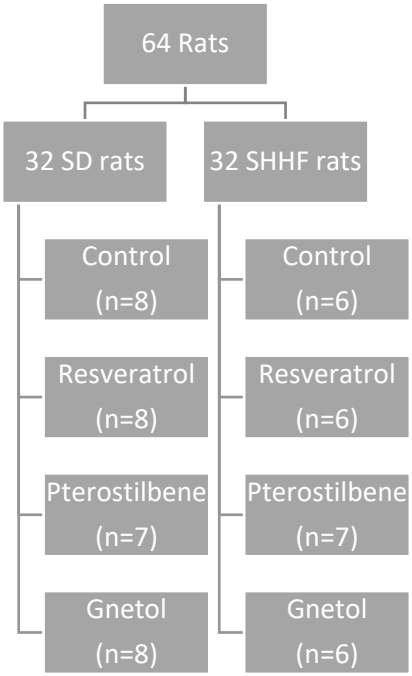



**Figure 28: Animal and treatment groups**

Each group of 8 animals was treated with the vehicle (control), resveratrol, pterostilbene or gnetol. There was some attrition due to premature death before the end of the study, reducing the total number of rats from 64 to 56. Figure 28 shows the remaining number of animals in each group by the end of the study. There was no remarkable pattern for the mortality observed within each treatment group.

### 6.1.3 Endpoint intraventricular septal thickness during systole and diastole

Intraventricular septal thickness is a measure of the size of the septum that separates the right and left ventricles and is an early indicator of ventricular hypertrophy. IVS was measured during both systole (IVSs) and diastole (IVSd) and normalized with the tibia length. Figure 29 shows the IVSs and IVSd dimensions with and without stilbenoid treatments. The dimensions were significantly higher within the SHHF rat groups compared to the SD control. Treatment with stilbenoids did not reduce IVSs and IVSd back to normal. Thus, stilbenoid treatment failed to attenuate ventricular hypertrophy in the SHHF rats.

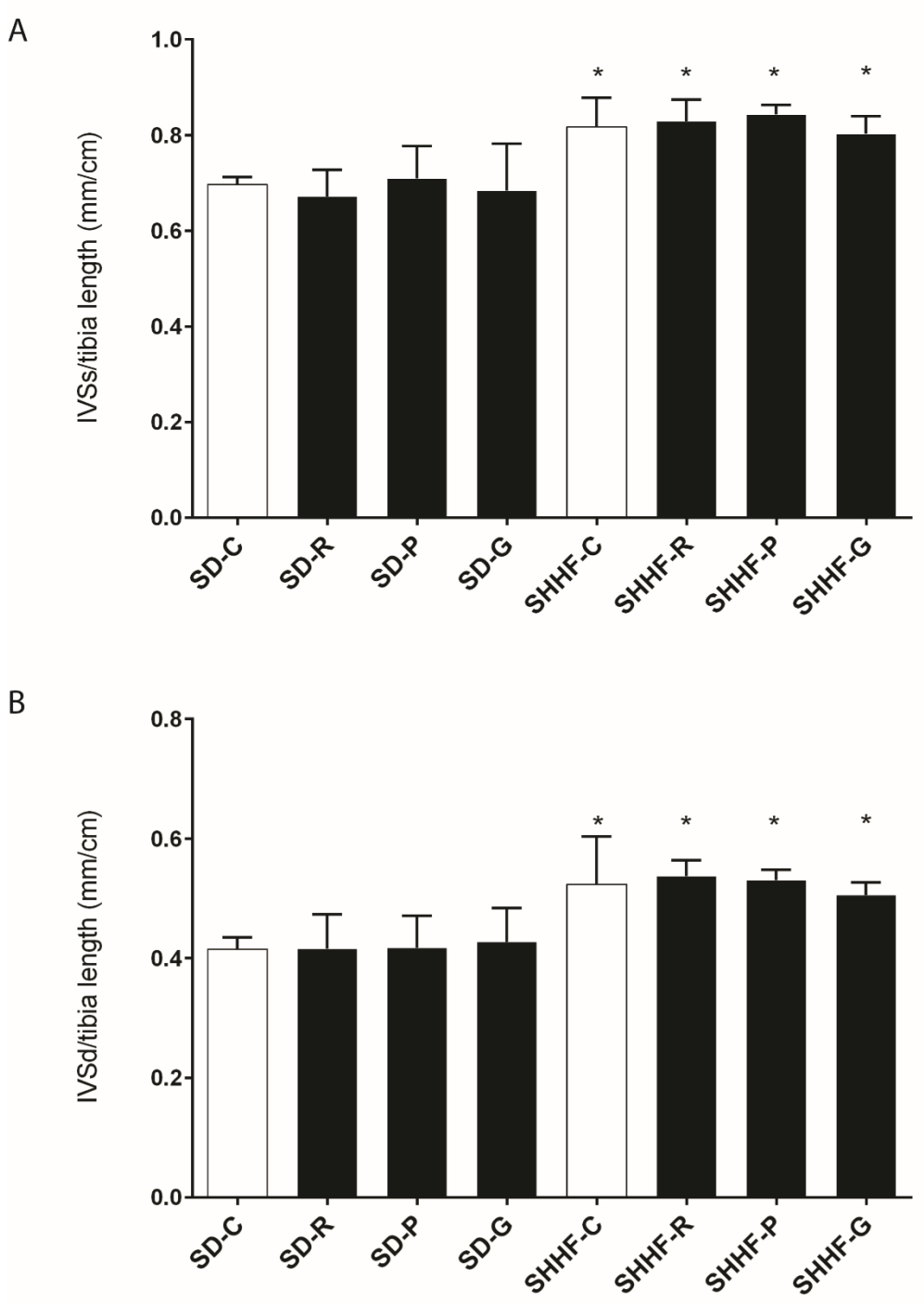



**Figure 29: Effects of stilbenoids on intraventricular septal thickness during systole and diastole**

Normalised A) IVSs and B) IVSd were significantly higher in the SHHF group than the SD group. Stilbenoid treatment did not prevent increases in A) IVSs and B) IVSd (n=6-8; *p<0.05 vs. SD-C).[466]

### 6.1.4 Baseline intraventricular septal thickness during systole

The baseline intraventricular septal thickness during systole (baseline IVSs), at the beginning of the study (age 9 weeks), was measured for both SHHF and SD controls. Figure 30 shows that the baseline IVSs was significantly increased in the untreated SHHF compared to the SD controls, suggesting pre-existing developing hypertrophy.

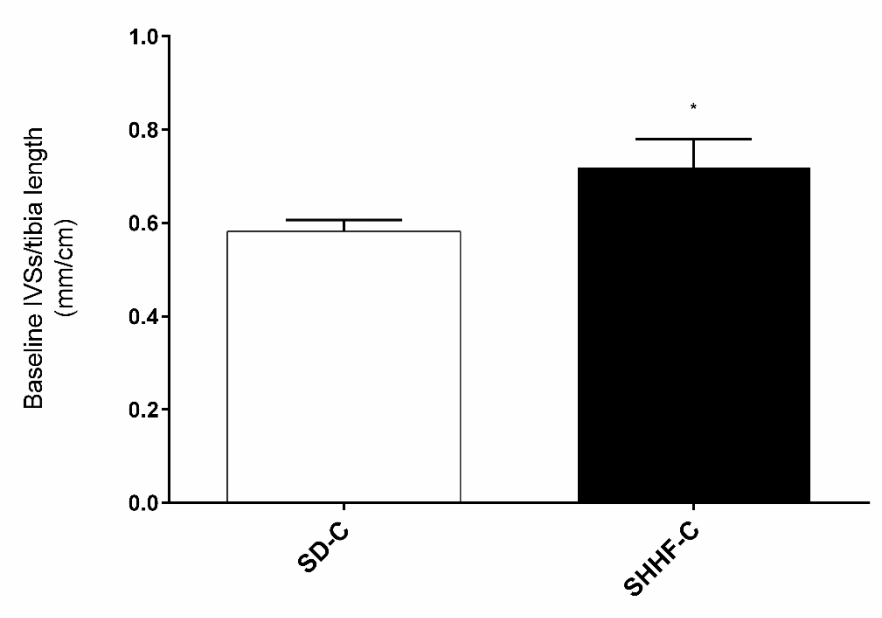



**Figure 30: Baseline intraventricular septal thickness during systole**

Normalized IVSs were significantly higher in the untreated SHHF (SHHF-C) compared to the age-matched untreated SD (SD-C) at the onset of the study, age 9 weeks (n=6-8; *p<0.05 vs. SD-C).

## 6.2 Cardiac function parameters

### 6.2.1 Systolic parameters: ejection fraction and fractional shortening

Ejection fraction is the fraction of the end-diastolic volume that is ejected during systole and is expressed as a percentage. This value is estimated by echocardiography. Fractional shortening is the fraction by which the end-diastolic dimension (EDD) is reduced during systole. Contraction leads to reduction of the EDD to end-systolic dimension (ESD). These dimensions are measured using M-mode echocardiography and the fractional shortening is estimated as EDD-ESD/EDD x 100. Both ejection fraction and fractional shortening are indicators of systolic function as they indicate how strongly the heart contracts.

The ejection fraction of the SHHF-C group was 75.8±4.4 % compared to 81.8±4.0 % in the SD-C group. Also, the fractional shortening was 39.3±4.0 % versus 45.8±4.4 % in the SD-C group. There was no statistically significant reduction in ejection fraction and fractional shortening in the untreated SHHF group compared to the untreated SD group (Figure 31).

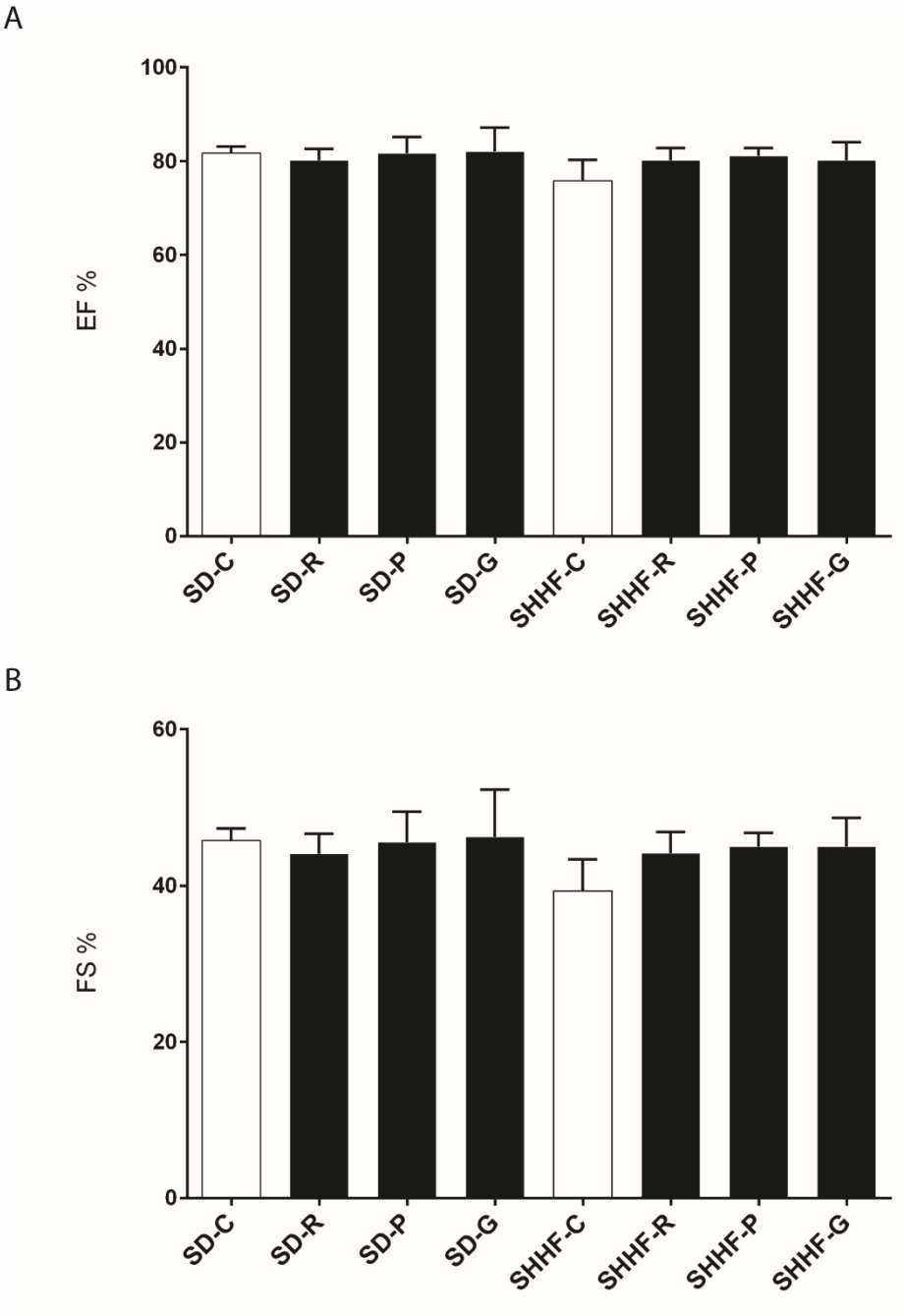



**Figure 31: Effects of stilbenoids on systolic parameters**

After 8 weeks of stilbenoid treatment, there were no significant differences in A) ejection fraction and B) fractional shortening between the SD and the SHHF groups (n=6-8).[466]

### 6.2.2 Diastolic parameter: iso-volumetric relaxation time

Isovolumetric relaxation time (IVRT) is the time required for iso-volumetric contraction to occur. This is a segment of the cardiac cycle where volumes of cardiac chamber remain constant while pressure and force of contraction is declining. IVRT is a key indicator of diastolic function since it indicates how long it takes the ventricular walls to relax during diastole. Prolongation of IVRT is an indication of diastolic dysfunction. Untreated SHHF rats exhibited longer IVRT (23.6±0.6 ms) compared to their age-matched SD control (16.6±1.4 ms). Treatments with resveratrol, pterostilbene and gnetol reduced IVRT to 19.3±1.3, 19.0±0.9 and 19.6±1.0 respectively (Figure 32). Thus, stilbenoid treatments improved diastolic function in SHHF rats.

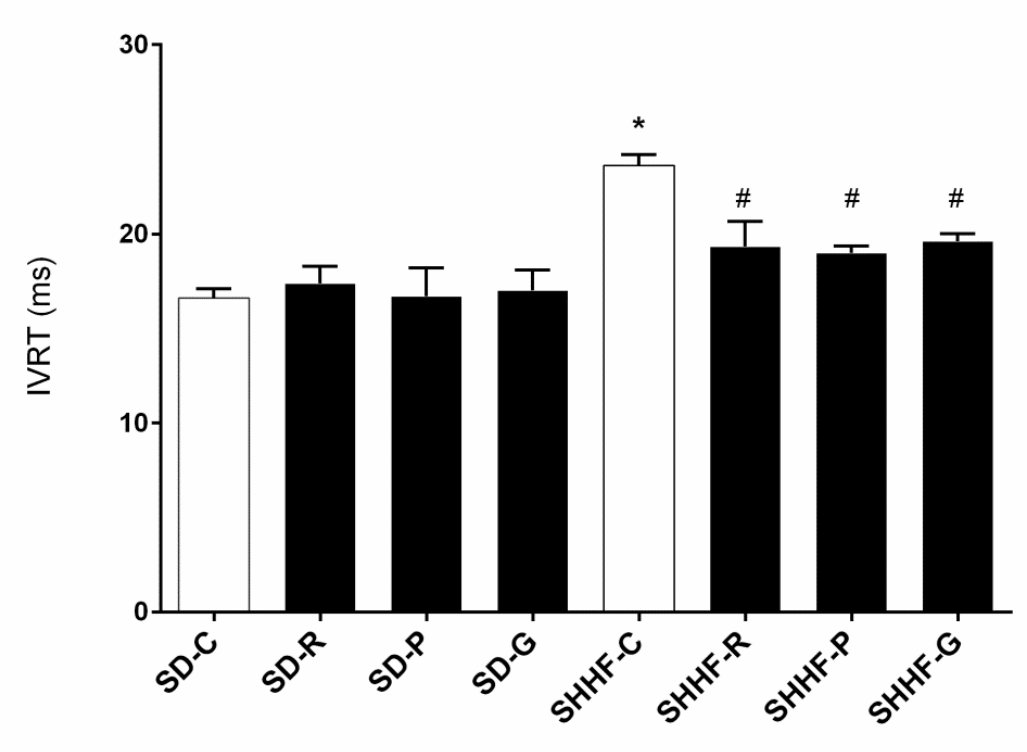



**Figure 32: Effects of stilbenoids on diastolic function- isovolumetric relaxation time**

Stilbenoid treatment prevented prolongation of IVRT in the SHHF group. Here we see a significant increase in the isovolumetric relaxation time in the untreated SHHF group, while the treated SHHF rat groups exhibited significantly lower IVRT values (n=6-8; *p<0.05 vs. SD-C; #p<0.05 vs. SHHF-C).[466]

### 6.2.3 Cardiac function - blood pressure

Stilbenoid treatments did not have significant effects on blood pressure in the SHHF rat groups (Table 3). Systolic blood pressures of the SHHF animals were already significantly higher at the onset of the experiment when they were 9 weeks old (Figure 33).

**Table 3: Effect of stilbenoids on blood pressure**

| Parameter (mm Hg) | SD | | | | SHHF | | | |
|---|---|---|---|---|---|---|---|---|
| | **C** | **R** | **P** | **G** | **C** | **R** | **P** | **G** |
| **Systolic BP** | 140±18 | 130±18 | 140±22 | 136±16 | 195±10[a] | 187±14[a] | 192±14[a] | 207±10[a] |
| **Diastolic BP** | 98±14 | 85±20 | 100±21 | 94±14 | 143±7[a] | 137±16[a] | 135±20[a] | 152±7[a] |
| **Mean BP** | 112±16 | 100±19 | 113±21 | 108±14 | 160±8[a] | 153±15[a] | 154±18[a] | 170±8[a] |
| **Pulse Pressure** | 43±5 | 46±5 | 41±5 | 42±4 | 52±6 | 50±7 | 56±9[a] | 54±4[a] |

C-control, R-resveratrol, P-pterostilbene, G-gnetol; (n=6-8); [a] $p<0.05$ vs. SD controls

Systolic, diastolic and mean blood pressures were higher in the SHHF group at the study endpoint (17 weeks old). Treatment with resveratrol, gnetol and pterostilbene did not result in significant lowering in the blood pressure.[466]

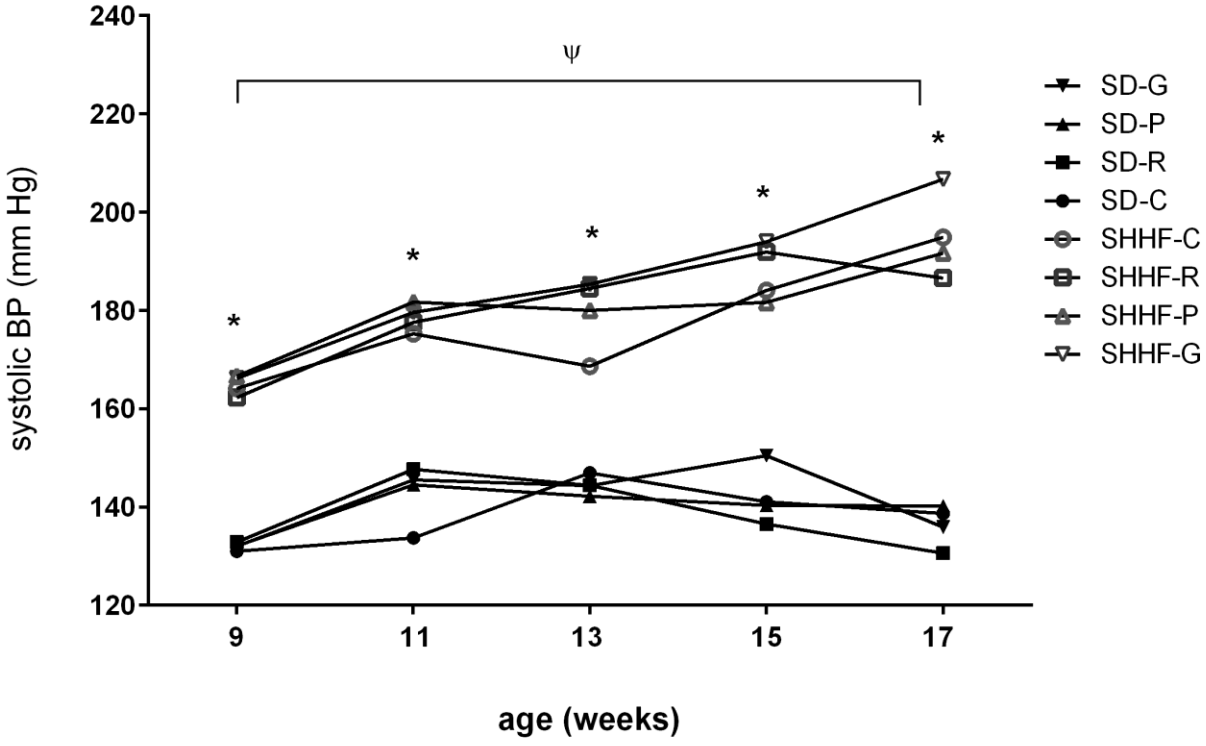



**Figure 33: Effects of stilbenoids on systolic blood pressure over time**

Systolic blood pressures of the SHHF rats (treated and untreated) were significantly higher than the SD controls and increased from week 9 to week 17. Stilbenoid treatment did not reduce the blood pressure. (n=6-8, *p<0.05 vs. SD controls, ψp<0.05 17 weeks vs. 9 weeks).

## 6.3 Hypertrophy signaling results

### 6.3.1 Levels of AMPK in heart tissues

Levels of AMPK protein, a possible hypertrophic signalling effector, were examined in heart tissue lysates from SD compared to SHHF rats. There were no significant differences in the levels of neither phosphorylated nor total AMPK (Figure 34).

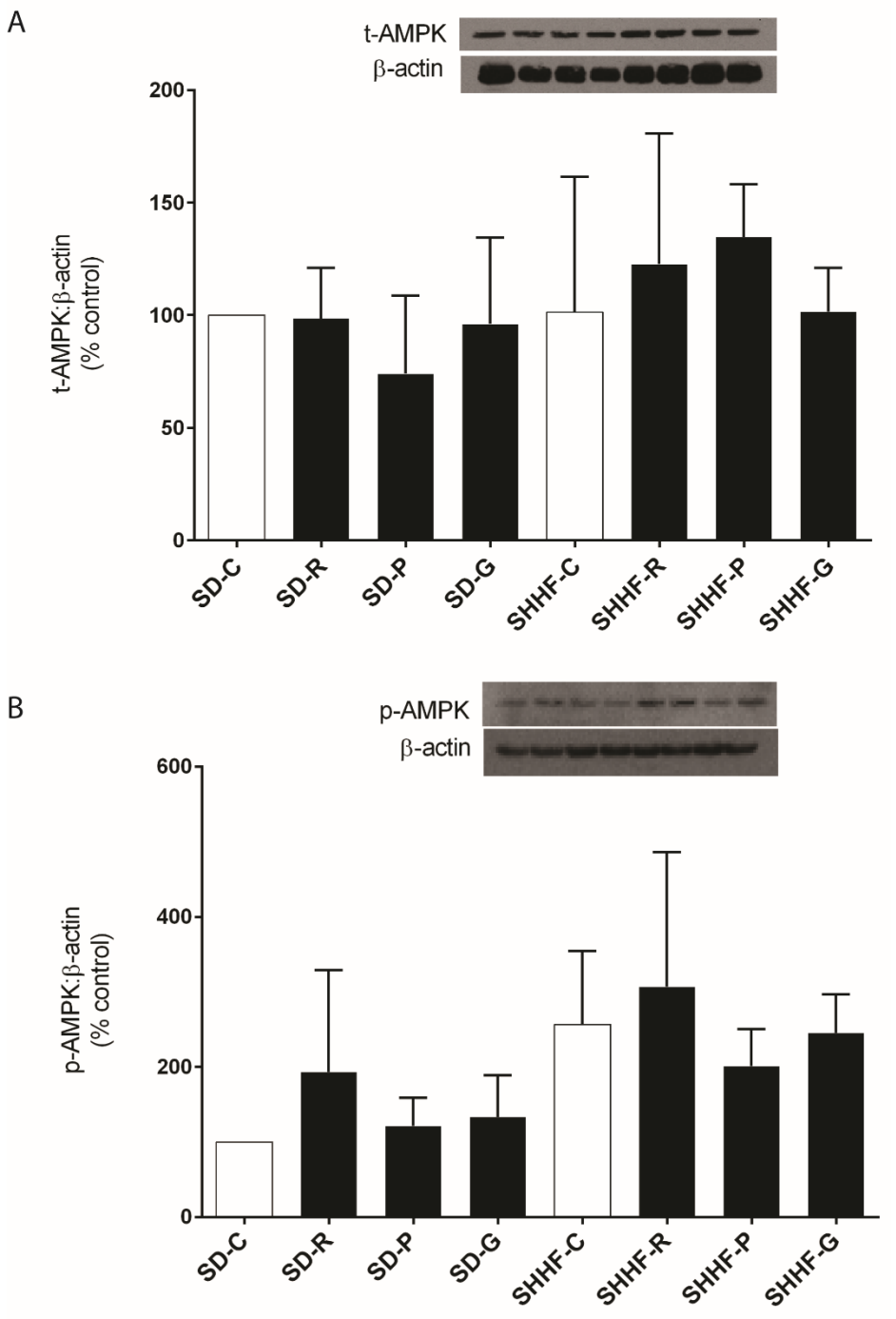



**Figure 34: Levels of AMPK activation in heart tissues with and without stilbenoid treatment**

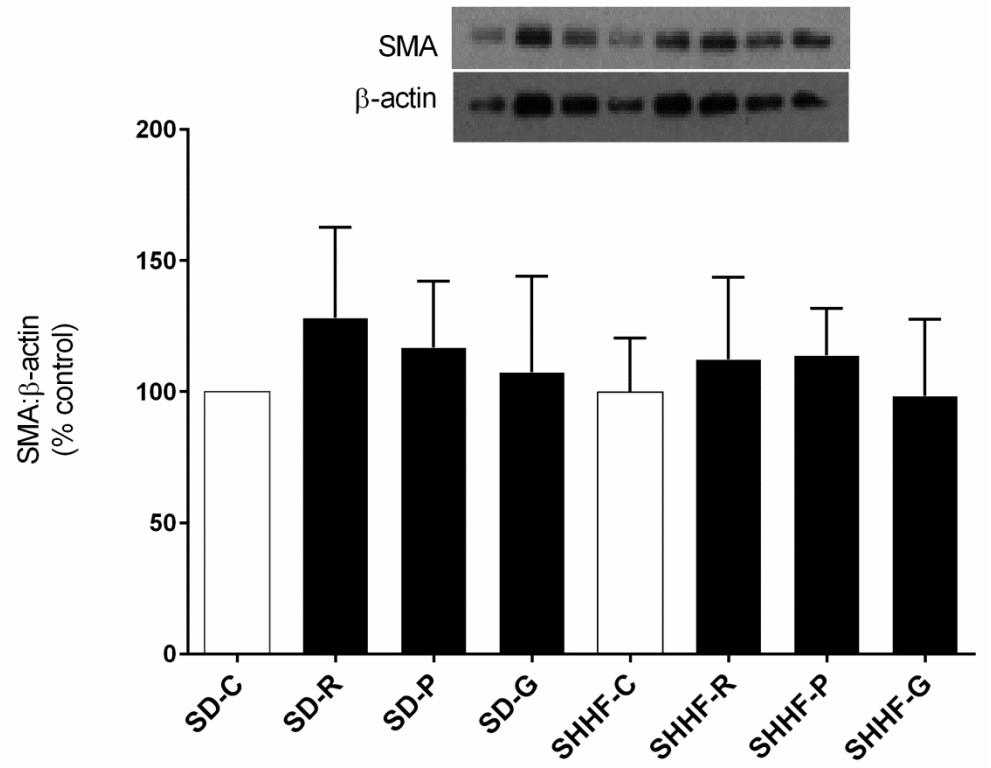
Ventricular tissue lysates were immunoblotted for p-AMPK and t-AMPK. There were no significant differences in the levels of A) t-AMPK and B) p-AMPK with and without stilbenoid treatment in SD and SHHF groups (n=5).[466]

## 6.4 Fibrosis markers

### 6.4.1 Smooth Muscle Actin (SMA) levels in heart tissue

There are many possible causes of diastolic dysfunction of which fibrosis may play a key role. During fibrosis, activation of fibrocytes to myofibroblasts is characterized by expression of α-SMA,[93] an actin isoform typically expressed in vascular smooth muscle cells.[467] There were no significant differences in the levels of SMA in between the SD and the SHHF groups (Figure 35).

**Figure 35: Levels of SMA in heart tissues with or without stilbenoid treatment**




Ventricular tissue lysates were immunoblotted for α-SMA protein levels. Stilbenoid treatment did not increase SMA protein levels in the SHHF rats group. There were no significant differences in the levels of SMA in SD and SHHF group with or without stilbenoid treatment (n=5).

### 6.4.2 Collagen I levels in heart tissue

Fibrosis is characterized by deposition of collagen in the extracellular matrix. The two predominant collagen types in the heart are collagen 1 and collagen III, which constitute about 80 % and 10 % respectively of total myocardial collagen.[468, 469] Thus, the levels of collagen 1 protein in the ventricular tissue lysate were measured by western blotting. Figure 36 shows that collagen 1 levels were reduced in the untreated SHHF rat compared to the SD control. Gnetol also reduced collagen 1 levels in the SD rat group compared to the SD control.

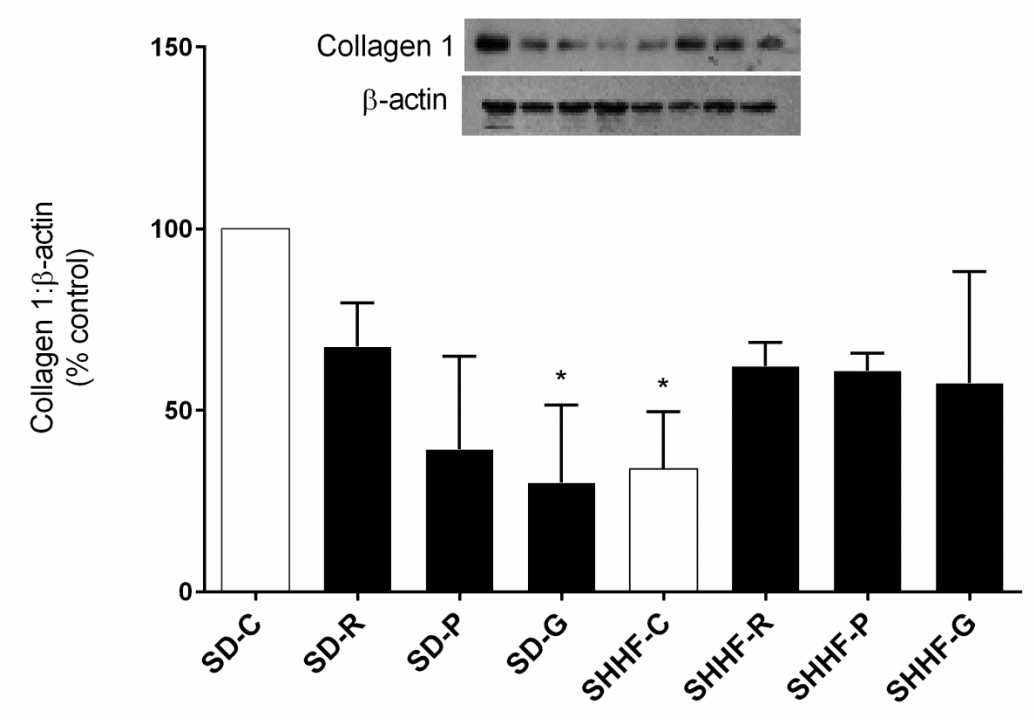



**Figure 36: Levels of collagen I in heart tissue with and without stilbenoid treatment**

Ventricular tissue lysates were immunoblotted for collagen 1 protein. Collagen 1 levels were significantly reduced in the untreated SHHF rats compared to SD control. Stilbenoid treatment did not significantly affect collagen 1 in the SHHF rat. Gnetol reduced collagen 1 in the SD rats group (n=3; *$p<0.05$ vs. SD-C).

# CHAPTER 7

# DISCUSSION

# 7.0 CHAPTER SEVEN: DISCUSSION

## 7.1 Anti-hypertrophic effects of stilbenoids *in vitro*

The effects of resveratrol on cardiomyocyte hypertrophy have been previously established in the literature. In cultured neonatal and/or adult rat cardiomyocytes, resveratrol inhibited cardiomyocyte hypertrophy induced by norepinephrine,[26] phenylephrine,[24] and Ang II.[470] In this project, the effect of resveratrol on ET-1-induced hypertrophy was tested in neonatal rat cardiomyocytes. Similar to previously reported data, resveratrol inhibited growth of cardiomyocytes induced by ET-1 at concentrations 1, 5, and 10 µg/mL (Figure 8). Higher concentrations of resveratrol (50 and 100 µg/mL) resulted in reduction of cell size and changes in cell morphology (cells appearing round) suggesting cell death. Higher concentrations of pterostilbene (5 - 10 µg/mL) and gnetol (50 - 100 µg/mL) resulted in significant reduction in cardiomyocyte viability. Dose-dependent activity of resveratrol has been demonstrated in the literature.[471] Increasing concentration of resveratrol (50 – 100 µM) induced apoptosis in endothelial cells.[472] The mechanism of death induced by high concentrations of stilbenoids may involve induction of apoptosis at high doses.

In cardiomyocytes, the maximum non-toxic concentration for pterostilbene (1 µg/mL) was 10-times lower than that of gnetol (10 µg/mL). This is consistent with previous data showing that pterostilbene is more potent than resveratrol.[433, 473, 474] Moreover, pterostilbene is more lipophilic due to the presence of methoxy- groups on the stilbene ring. Higher lipophilicity may increase the rate and extent of uptake into myocytes and thus enhance its activity even at lower concentrations.

Effects of gnetol and pterostilbene on hypertrophic markers

The effects of gnetol and pterostilbene on cardiomyocyte hypertrophy have not been previously explored. Here, we showed for the first time that gnetol inhibited all three markers of hypertrophy

while pterostilbene blocked two out of three hypertrophic markers. Gnetol prevented cardiomyocyte enlargement, protein synthesis and BNP induction in cultured isolated neonatal rat cardiomyocytes (Figure 10). This is consistent with a previous report, in which resveratrol decreased protein synthesis and β-MHC promoter activity in Ang II-induced cardiomyocyte hypertrophy.[470] Furthermore, pterostilbene prevented cardiomyocyte enlargement and protein synthesis but did not prevent BNP induction.

Although fetal gene induction is widely considered a hallmark of hypertrophy, the role of activation of fetal genes during the hypertrophic response is somehow debated.[475] There are two propositions regarding fetal gene induction during hypertrophy. On one side, activation of the fetal gene program may be an adaptive response which helps to combat metabolic changes that occurs during hypertrophy.[73, 476] Alternatively, the effects of fetal gene induction may be deleterious and may contribute to further disease progression.[477-479] The ability or inability of stilbenoids to inhibit BNP expression may not confer superiority on one agent over the others. The potential anti-hypertrophic effects of pterostilbene should not be ignored based on inability to block BNP expression in cardiomyocytes.

Although hypertrophic growth is mostly associated with enhanced fetal gene expression, findings by Patrizio *et al.* showed that mechanisms involved in cardiomyocyte growth and fetal gene expression are distinct and independent.[480] Propranolol inhibited cardiac hypertrophy in the aortic-banded pressure overload rat model of hypertrophy.[481] Patrizio *et al.* also reported that propranolol inhibited cardiac hypertrophy in thoracic aortic coarctation mice model but increased expression of fetal genes such as ANP and β-MHC.[480] Cardiac hypertrophy was measured by left ventricular weight to body weight ratio and cross-sectional area of cardiomyocytes in left ventricular sections.[480] Propranolol treatment also increased fetal gene expression in phenylephrine-treated

neonatal rats cardiomyocytes.[480] Thus, it is possible to inhibit cardiomyocyte growth without downregulation of fetal gene expression.

The effect of pterostilbene on BNP expression is similar to the previous report for propranolol. This suggests that pterostilbene inhibited signaling involved in cardiomyocyte growth and protein synthesis (AMPK-mTOR-Akt) but had no effects on mechanisms involved in fetal gene expression. For propranolol, authors proposed that fetal gene induction was mediated by β1-adrenoceptor blockage since this effect was disrupted by treatment with prazosin.[480] ET-1 induces hypertrophy in cardiomyocytes via activation of GPCR (ET-1 receptors) and subsequent activation of ERK1/2. ET-1 induces fetal gene expression via PKC activation and increased expression of IL-6 cytokine.[482] ET-1-induced BNP expression is significantly suppressed by PKC inhibitors and tyrosine kinase inhibitors.[483] It is possible that pterostilbene was unable to inhibit signaling mechanisms involved in fetal gene expression in cardiomyocytes, such as such PKC activation and IL-6 expression.

## 7.2 Anti-hypertrophic signaling of stilbenoids *in vitro*

### 7.2.1 AMPK signaling

Stilbenoids activate AMPK

AMPK is activated by phosphorylation at the threonine-172 residue by upstream LKB1 or CAMKKβ.[219-221] During low energy conditions and metabolic stress, phosphorylation of AMPK conserves energy by shutting down energy-consuming processes, such as protein synthesis, and enhances energy-generating processes such as glucose uptake, fatty acid uptake and glycolysis.[236] Pharmacological activation of AMPK by metformin and AICAR inhibited phenylephrine-induced protein synthesis in neonatal rat cardiomyocytes and Ang II-induced cardiomyocyte hypertrophy.[232] Previous studies have also shown that resveratrol inhibits cardiomyocyte

hypertrophy via activation of AMPK.[24, 26] This is consistent with findings that gnetol and pterostilbene activated AMPK via phosphorylation at specific time points.

Although gnetol did not significantly increase total AMPK levels, gnetol increased phosphorylated AMPK levels transiently, at the 4-hour time point, and phosphorylated AMPK levels returned to basal levels after 24 h (Figure 14). Pterostilbene increased levels of both total AMPK (8 and 24 h) and phosphorylated AMPK (4, 8, and 24 h). Pterostilbene had a more sustained effect on AMPK activation (Figure 17). Overall both gnetol and pterostilbene activated AMPK at least at one specific time point. Transient activation of AMPK by gnetol is a common phenomenon with signaling cascades, in which activation of one signaling molecule leads to the activation of other signaling molecules downstream. For example, ET-1 induces phosphorylation of ERK1/2 within 10 min of exposure to cardiomyocytes while p-ERK1/2 returns to basal levels after 30 min until 24 h.[484] Despite the return of p-ERK1/2 to basal levels, ERK activity was sustained and hypertrophic phenotype was detected at 24 h after ET-1 treatment.[484] Thus, although AMPK activation by gnetol was not sustained, transient activation may be sufficient to elicit appropriate downstream signaling. Furthermore, Lu *et al.* reported similar transient phosphorylation of AMPK and eNOS by CB-13, a synthetic cannabinoid receptor agonist.[485] Phosphorylation of eNOS may lead to increased NO production. NO has been reported to inhibit ET-1-induced cardiomyocyte hypertrophy via inhibition of the RhoA/ROCK signaling, an early effector of hypertrophy.[486] The effect of stilbenoids on RhoA/ROCK signaling in cardiomyocytes, with respect to their anti-hypertrophic effects, is yet to be determined.

Stilbenoids inhibit hypertrophy via AMPK activation

In the presence of compound C, both gnetol and pterostilbene were unable to inhibit cardiomyocyte growth. This suggests a role for AMPK in the anti-hypertrophic effects of gnetol and pterostilbene.

Although compound C reportedly inhibits AMPK at an $IC_{50}$ of 0.1 - 0.2 μM, it also inhibits other protein kinases such as FGF-R1, ERK8 and MNK1.[487, 488] Furthermore, compound C inhibited BMP and VEGF at concentrations as low as 0.1 μM.[488] Therefore compound C is not considered specific to AMPK but it remains useful as an initial screening of the possible role of AMPK. The role of AMPK was further confirmed by knocking down the catalytic subunit of AMPK using lentiviral constructs expressing shRNA against AMPKα1/2 isoforms.

Similar to the effect of compound C, the anti-hypertrophic effects of both gnetol and pterostilbene were lost when AMPKα gene was knocked down. Since genetic knockdown is a more specific way of targeting a gene of interest, it can be inferred that gnetol and pterostilbene inhibit cardiomyocyte hypertrophy, at least in part, via activation of AMPK. This result is consistent with existing data regarding resveratrol. Thandapilly *et al.* reported that resveratrol inhibits norepinephrine-induced hypertrophy via activation of NO-AMPK signaling.[26] Increased NO produced may exert anti-hypertrophic effects via inhibition of RhoA/ROCK signalling in cardiomyoytes.[486]

Levels of AMPKα1 and AMPKα2 protein isoforms were reduced to 29.6 % and 35.2% respectively (Figure 20). It was observed that AMPK α2 isoform was more difficult to silence. This is consistent with data from literature showing that AMPK α2 is the more predominant isoform in the heart. [217, 218]

### 7.2.2 MITF signaling

The role of MITF in cardiac hypertrophy is only emerging and not well understood. Mutation of the MITF gene in transgenic mouse models reduces the hypertrophic response to Ang II and isoproterenol.[267, 268] Thus, it is believed that MITF is required for cardiac hypertrophy.[267, 268]

ML329, an MITF inhibitor, blunted the hypertrophic response to ET-1 in neonatal rat cardiomyocytes. This further supports the role of MITF in cardiac hypertrophy. ET-1 increased MITF expression at 30 min and 4 h time point in neonatal rat cardiomyocytes (Figure 22). This is consistent with previous studies that showed that ET-1 increased MITF expression in melanocytes[458, 459] and that isoproterenol increased MITF expression and protein levels in cardiomyocytes.[270]

The time-course effects of gnetol and pterostilbene alone on MITF expression revealed no significant effect on MITF protein levels over a period of 24 h. Since ET-1 increased MITF expression, the effect of stilbenoid pre-treatment on ET-1-induced MITF expression was examined. Pre-treatment with gnetol and pterostilbene prevented increase in MITF expression, when challenged with ET-1. This means that gnetol and pterostilbene prevented MITF expression induced by ET-1 treatment. However, it can not be concluded that stilbenoids inhibited hypertrophy via MITF downregulation. Further studies that involve treatment of cardiomyocytes overexpressing MITF with stilbenoids may shed further light to the role of MITF in the anti-hypertrophic effects of stilbenoids.

Unlike gnetol and pterostilbene, resveratrol increased MITF levels at 4 h in the presence and absence of ET-1. This contrasts previous studies where resveratrol inhibited MITF activity and protein expression in melanoma cells,[489] and had no effect on MITF protein levels in normal human melanocytes.[490] The implication of the disparity between the effects of resveratrol on MITF in cardiomyocytes versus melanocytes is not clear and warrants further study.

## 7.3 AMPK – MITF crosstalk

Since stilbenoids prevented ET-1 induced hypertrophy via activation of AMPK, it was speculated that AMPK activation may be involved in the regulation of MITF. There was no increase in MITF

levels after 4 h of ET-1 treatment in the presence of compound C. This suggests that AMPK inhibition represses MITF protein levels in the presence of hypertrophic stimuli (ET-1). This is consistent with previous studies that showed downregulation of MITF with compound C treatment.[274] Interestingly, AMPK inhibition did not inhibit hypertrophy as shown by hypertrophic response to ET-1 even with compound C pre-treatment (Figure 15).

If MITF is required for hypertrophy, then it must act on certain targets to fulfil this effect. MITF may act as a co-activator of other hypertrophic signalling pathways. As a transcription factor, MITF binds to the E box motif of target DNA containing the canonical CATGTG sequence to promote transcription of target genes.[264] Target genes include those involved in melanin synthesis (TRY, TRYP1), apoptosis (bcl-2) and melanocyte proliferation (CDK).[262] Other downstream targets of MITF related to cardiac hypertrophy include: erbin,[269] GATA,[270] and miR-541.[268] MITF increased expression of Erbin, a negative regulator of hypertrophy, under basal conditions but repressed Erbin during isoproterenol-induced hypertrophy.[269] MITF regulates cardiac hypertrophy by interacting with miR-541 and repressing its transcription.[268] Furthermore, MITF binds with the E-box element of the GATA4 promoter to activate expression of the pro-hypertrophic transcription factor, GATA4, in response to β-adrenergic stimulation.[270] These targets of MITF may be studied to better understand the regulation of MITF in cardiomyocyte/cardiac hypertrophy.

## 7.4 Effects of stilbenoids on hypertrophy *in vivo*

Stilbenoid treatments did not significantly reduce blood pressure in the SHHF rat model. This observation is, in fact, not surprising as previous studies have shown that low dose resveratrol attenuates cardiac hypertrophy without significant effect on blood pressure.[319, 322] In other studies however, reduction in blood pressure was achieved by administration of higher doses of resveratrol.[320, 362] In this study, a slight reduction in systolic blood was observed for resveratrol

(8.3 mm Hg). Although this effect was not statistically significant, it may have some clinically significant implications. In humans, epidemiological studies have shown that graded reduction in blood pressure correlates to lower risk of cardiovascular disease events and all-cause mortality. For every 10 mm Hg-reduction in systolic blood pressure, all-cause mortality was reduced by 13%.[491] Indeed, systolic blood pressure reduction as low as 5 mm Hg resulted in 14% reduction in stroke and 9% reduction in coronary heart disease.[492] Thus the slight reduction in systolic blood pressure by stilbenoids may be clinically significant.

The systolic function of SHHF rats was not significantly impaired at the endpoint of the study. At this stage (17 weeks old), significant decline in systolic function was not expected since the rats were still in the early stage of the disease timeline. This is consistent with previous studies showing that decompensation of systolic function does not occur until later stages of disease (15 months old).[441]

Among hypertrophic markers examined, hypertrophy was observed only in the septum as shown by a significant increase in IVSs and IVSd thickness measured both at systole and diastole respectively. Although stilbene treatments improved diastolic function by reducing IVRT, stilbenes did not reduce hypertrophic markers (IVSd and IVSs) back to normal. There are a few possible explanations for this phenomenon.

Pre-existing hypertrophy

First, it may be possible that the intraventricular septal hypertrophy was present at the beginning of the study prior to stilbenoid treatment. In this case, pre-existing hypertrophy may be more difficult to reverse (as opposed to prevention). At the onset of the experiment, 9-week-old SHHF rats already had high average systolic blood pressure of 164 ± 18 mm Hg compared to 134 ± 18

mm Hg in age-matched SD controls. Indeed, the baseline echocardiographic measurements show that at 9 weeks of age, the SHHF rats already exhibited significantly increased IVSs compared to the SD control (Figure 30). In humans, hypertrophy limited to the septum has been observed in patients with high blood pressure and is termed asymmetric septal hypertrophy. This has been associated with increased risk of atrial fibrillation in patients with hypertrophic cardiomyopathy.[493]

Early hypertrophy

Second, the fact that hypertrophy was only observed in the septum and not in other ventricular dimensions may indicate that the animals were in an early stage of hypertrophy. Normalized dimensions of other parts of the heart such as the left ventricular posterior wall thickness, left ventricular internal diameter, relative wall thickness, estimated left ventricular mass, and the whole heart weights remained similar to the SD controls. Previous research has shown that the septum is more susceptible to hypertrophy.[494, 495] Therefore, hypertrophy may first be observed in the septum and then may progress to other parts of the heart as the condition worsens. Thus, it may be possible that during hypertrophy the deleterious signaling pathways that are characteristic of pathologic hypertrophy have not yet been activated. Dolinsky *et al.* showed that resveratrol prevents pathologic but not physiologic hypertrophy.[496] It is possible that the stilbenes do not block signaling mechanisms involved in early compensated hypertrophy such as phosphorylation of Akt and p70S6K.

Pathologic to physiologic switch

An alternate explanation is that perhaps stilbenoids stimulated a switch from pathologic to physiologic hypertrophy. Exercise training can cause such a switch in SHR.[497] Swimming exercise protocols in SHR reduced collagen volume fraction, improved myocardial capillary density and enhanced systolic function, while reducing markers of pathologic hypertrophy such as

calcineurin/NFAT activity, ANF and MHC mRNA abundance.[497] There are a few commonalities between exercise-induced and stilbenoid treatment-induced signaling. For example, resveratrol induced mitochondrial biogenesis via activation of PGC-1α.[415] Similarly, endurance exercise up-regulates PGC-1, leading to improved mitochondrial function and increased myocardial antioxidant enzymes such as superoxide dismutase.[498] These similarities suggest that stilbenoid treatments may as well induce a change from pathologic to physiologic hypertrophy.

To explore this hypothesis, it may be helpful to examine markers of physiologic hypertrophy (IGF-PI3K-AKT) pathway and markers of pathologic hypertrophy (MAPK-ERK) pathway. The IGF-PI3K-AKT is one of the best characterized pathways involved in physiologic hypertrophy. Cardiac levels of IGF-1, but not ET-1 or Ang II, are higher in professional soccer players compared to non-athletic controls.[201] Serum levels of IGF-1 were also increased after intense exercise training in humans[202] and in rats, [203] indicating the role of IGF-1 signalling in physiologic hypertrophy. Moreover, the downstream targets of IGF-1 such as PI3K and AKT levels were elevated in the transgenic mouse hearts overexpressing IGF-1, while signaling molecules known to be involved in pathologic hypertrophy such ANP and BNP remained inactivated.[204]

The MAPK signaling pathway can be classified into 3 main categories: ERK1/2, p38 kinases and c-Jun N-terminal kinases (JNK).[499] While activation of p38 and JNK is mostly associated with inflammation and stress responses, activation of ERK1/2 is closely related to cell growth as it may occur in pathologic cardiac hypertrophy.[500] ERK1/2 is activated by an upstream kinase, MEK1/2.[501] Pathologic hypertrophic stimuli such as pressure overload lead to activation of MEK-ERK pathway. Activity of ERK1/2 was increased in 6 months old SHHF rats.[502] Activities of MAPK (p38, JNK and ERK1/2) were also increased in other models of hypertrophy and hypertension.[503, 504] This supports the role of ERK1/2 in pathologic hypertrophy.

The effect of stilbenes on the conversion of pathologic to physiologic hypertrophy has not been previously explored and warrants further study. If this happens, markers of physiologic hypertrophy may be activated in stilbenoid-treated SHHF while remaining normal in the untreated and in the age matched SD control groups. Conversely, markers of pathologic hypertrophy may be activated only in the untreated SHHF group.

## 7.5 Anti-hypertrophic signaling *in vivo*

Although AMPK was activated by stilbenoids and implicated in the anti-hypertrophic effects of stilbenoids *in vitro*, AMPK did not seem to play a role *in vivo* in SHHF rats. This may be due to differences between the experimental settings *in vitro* and *in vivo*. In the *in vitro* experimental setting, stilbenoids acted directly on cardiomyocytes indicating specific cellular responses. However, when administered *in vivo*, stilbenoids act on a more complex system involving not only cardiomyocytes, but other cell types in the heart and other components of the cardiovascular system. Thus, it may be more difficult to obtain the same effect (AMPK activation) as the *in vitro* setting. Different responses may also be obtained in different experimental models (e.g. SHR vs. SHHF).

As a model of heart failure, SHHF rats normally exhibit eccentric hypertrophy in contrast to SHR, which exhibit concentric hypertrophy.[505] Resveratrol inhibited concentric hypertrophy in aortic banding-induced pressure overload model, but not eccentric hypertrophy in aortocaval shunt-induced volume overload.[360] Cardiac AMPK is reduced in the SHR and activation of AMPK by resveratrol has been shown to inhibit hypertrophy in both isolated cardiomyocytes and in the SHR.[26, 249] However, in the current study, AMPK levels remained unaffected in the SHHF rat heart at 4 months (Figure 34). Neither resveratrol, gnetol nor pterostilbene significantly activated AMPK (Figure 34) in SHHF rats. Although metformin activated AMPK in SHHF rats and upregulated

cardiac eNOS protein levels, this was not accompanied by significant reduction in heart weight to bodyweight ratio and cardiomyocyte diameter.[506]

eNOS is a possible signalling molecule downstream of AMPK.[507] Upregulation of eNOS expression and calcium-dependent NO production via AMPK activation attenuates of cardiac hypertrophy.[508, 509] Cardiac eNOS protein expression is unchanged in ET-1-treated cardiomyocytes[486] and reduced in pressure overload-induced hypertrophy.[25] However in SHHF rats, cardiac eNOS is increased compared to age-matched SD and SHR at 18 months of age.[510] Thus, further upregulation of eNOS expression subsequent to AMPK activation may not exert anti-hypertrophic effects in SHHF. This implies that activation of AMPK may not be required for anti-hypertrophic effects in the SHHF rat model and thus suggests a significant difference in AMPK signalling between the SHR and SHHF rat models.

Improvement of systolic and/or diastolic function may occur even in the presence of cardiac hypertrophy in the SHHF rat.[466, 506] Rimbaud *et al*. showed that resveratrol improved survival and preserved cardiac function without significant reduction in hypertrophy or blood pressure in the Dahl salt-sensitive rat model of heart failure.[455] Similar to our data, AMPK was neither activated nor repressed in the Dahl-salt sensitive rats at 17 weeks of age. Furthermore, resveratrol improved diastolic function without affecting hypertrophy in a pressure-overload-induced TAC mouse model of heart failure.[511] In this model, activated AMPK levels were reduced in the untreated TAC group compared to the sham control and resveratrol treatment increased AMPK activation back to normal.[511] Thus, the overall beneficial effect of stilbenoids on cardiac function extends beyond possible anti-hypertrophic effects and should not be ignored.

Moreover, stilbenoids are phytoestrogens and thus may exert cardioprotective effects via activation of estrogen receptors.[512] Resveratrol binds to estrogen receptors at concentrations comparable to those needed for other biological activities.[512] Endogenous estrogen is known to be cardioprotective, since the risks of cardiovascular diseases increases in post-menopausal women and oophorectomized young women.[513] The possible role of estrogenic properties of stilbenoids in their cardioprotective effects is yet to be ascertained.

It is important to note that relatively low doses of the stilbenoids were administered within a short time frame (8 weeks). It is possible that longer treatment duration even at similarly low dose (2.5 mg/kg/day) may result in better outcomes in the disease progression. For instance, Ahmet *et al*. reported that long-term (10 months) administration of low dose resveratrol (5 mg/kg/day) resulted in improved cardiac function in a post-myocardial infarction model of heart failure in rats.[514] Improvement in cardiac function was not evident until 4 months treatment.[514] This suggests that long-term, low dose resveratrol, similar to doses obtainable from natural sources, may provide additional benefit in heart failure disease states. Clinical trials to test the role of resveratrol in heart failure patients are needed in the future.

## 7.6 Anti-fibrotic signaling *in vivo*

Effect on interstitial fibrosis

One of the major contributors to diastolic dysfunction is the development of fibrosis and ventricular stiffness.[515] Improvement of diastolic dysfunction by stilbenoids indicates possible effects on fibrosis. Fibrosis is normally a component of cardiac remodeling and occurs as a reparative response to injury or stress. While cardiac hypertrophy involves changes in cardiomyocytes, fibrosis involves changes in the extracellular matrix. Cardiac fibrosis begins with increased deposition of extracellular matrix proteins such as collagen I and fibronectin in the

extracellular matrix.[516] In the later stages, myofibroblasts become activated and they produce increased levels of α-SMA.[517]

There are two types of fibrosis: interstitial fibrosis and replacement fibrosis.[93, 518] In interstitial fibrosis, increased deposition of extracellular matrix proteins occurs in areas of injury as well as in otherwise healthy parts of the heart tissue.[497] This results in increased mechanical stiffness of ventricular walls, inability of the ventricles to relax properly and prolongation of relaxation time.[515] Replacement fibrosis occurs in the advanced stage or due to loss of cardiomyocytes post-myocardial infarction. At this point the systolic function would have been impaired. In order to improve systolic function, expression of α-SMA in activated myofibroblasts confers contractile activity on the myofibroblasts.[519]

In the case of the SHHF rats (4 months old), systolic function was not yet impaired. That is, the ejection fraction and the percent fractional shortening were not significantly different from the SD controls (Figure 31). This is consistent with previous data that shows systolic decompensation in SHHF rats does not occur until 15 months of age.[441] Thus we may infer that fibrosis may be in its early stage and limited to the interstitial levels at age 4 months when our experiment was terminated. In fact, α-SMA levels remain similar in both SD and SHHF animals. This suggests that complete differentiation of myofibroblasts and tissue injury or scarring have not yet occurred. This is also consistent with previous characterization of the SHHF animal that showed fibrotic lesions were not evident until 9 months of age.[441]

Resveratrol inhibits proliferation of fibrocytes *in vitro*[520] and also prevents collagen deposition by cardiac fibroblasts *in vivo.*[521] Both resveratrol and pterostilbene inhibit markers of fibrosis in rat liver.[522, 523] Therefore, I hypothesized that improvement in diastolic function by stilbenoids may be due to reduction in collagen production and deposition in the extracellular matrix. However,

measurement of collagen I protein in the left ventricular tissues showed lower levels of collagen I in the untreated SHHF rats, while collagen I levels remained unchanged with stilbenoid treatment in SHHF rats (Figure 36). This contrasts with the general proposition that increased collagen deposition is associated with diastolic dysfunction.

Previous characterization of SHHF rats showed that although collagen volume fraction of the extracellular matrix increased, expression (mRNA levels) of collagen I and III in the heart of SHHF rats remained low from age 4 months until 18 months.[441] There is also evidence that matrix metalloproteinases (MMP) expression is increased in SHHF rats[441] and indeed treatment with inhibitors of MMP improves LV dysfunction and remodelling.[524, 525] High MMP expression, which may be due to increased levels of cytokines such as IL-1 and TNF-α, may result in collagen degradation.[526] SHHF rats produced significantly higher TNF-α than SHR and SD controls at age 18 months.[527] The extent of collagen degradation is balanced by actions of an endogenous MMP inhibitor, tissue inhibitor of metalloproteinases (TIMP).[528] Furthermore, transient degradation of collagen has been reported in other cardiac disease models such as post-myocardial infarction,[529] stunned myocardium,[530] and dilated cardiomyopathy.[531] Alteration of the extracellular matrix is associated with ventricular dilation in response to volume overload and eccentric hypertrophy.[532] Since SHHF is a model of dilated cardiomyopathy and involves eccentric hypertrophy,[505] the lower collagen levels observed here may be an early response in the remodelling of SHHF ventricle.

Similar to collagen I and III, fibronectin mRNA levels remained unchanged in SHHF rat ventricles from age 4 months to 18 months despite progression in myocardial fibrosis.[441] This suggests that fibronectin may not be a better marker of fibrosis in SHHF rats. Thus, fibronectin levels were not examined in this study.

## 7.7 Bioavailability and bioactivity of stilbenoids

Despite the widely demonstrated beneficial effects of resveratrol, the optimal dose that would be effective in humans is yet to be established.[533, 534] Studies in cardiomyocytes utilized concentrations of resveratrol that range between 10-60 µM.[26, 466, 535, 536] Due to low bioavailability, it is difficult to achieve such resveratrol concentrations (µM range) in animal and human plasma after oral administration. For instance, administration of 500 mg and 5 g of resveratrol to healthy humans yielded maximum plasma concentrations of 72.6 ng/mL and 538.8 ng/mL respectively,[537] whereas plasma levels of resveratrol glucuronide and resveratrol-3-sulphates with 500 mg dosing were 404.6 and 1135 ng/mL respectively.[537] Further increase in dose and repeated multiple doses did not result in increased peak plasma concentration of resveratrol.[538] There is high inter-individual variation in the pharmacokinetics of resveratrol in humans.[537, 538] This may be due to variations in rate and extent of metabolism among subjects in the studies.

Although some human clinical studies have shown biological effects of resveratrol, the role of bioavailability on bioactivity is still not clearly understood. In this project, equal doses of three stilbenoids were administered: pterostilbene with high bioavailability (80%),[29] resveratrol with low bioavailability (29.8%)[296] and gnetol with even much lower bioavailability (6.59%).[295] Based on the bioavailabilities, varied responses were expected from the administration of these compounds. However, there were no differences among responses observed. In fact, all three stilbenoids improved diastolic function to the same extent. This implies that they reached heart tissue in sufficient concentrations to elicit the biological effect. Thus, it may be inferred that bioavailability is not necessarily a limitation for the biological activity of stilbenoids. Other factors such as role of metabolites must be considered.

One major reason for low bioavailabilities of stilbenoids is rapid and extensive metabolism.[295, 303] Pterostilbene has two methoxy- substituents making it more resistant to metabolism and thus more bioavailable.[29] Gnetol and resveratrol exerted biological activities despite low bioavailabilities. This is possibly due to reconversion of sulphates and glucuronide metabolites back to free drugs by action of sulphatases and glucuronidases at the site of action.[305, 307] Enterohepatic cycling may also play a role by increasing the duration of action of stilbenoids since they stay longer in the body.[296, 303] Lastly, it is possible that some metabolites are biologically active.[304] For example resveratrol glucuronide exhibited almost equal anti-inflammatory and anti-oxidant activities as the parent compound.[306] Moreover, resveratrol-4-sulphate inhibited COX-1 and COX-2, and activated SIRT1 with a potency similar to resveratrol.[304] Thus, the metabolites may be active and/or form a pool of constant supply of parent drug.

The role of metabolites in the activities of stilbenoids is not clearly understood. Inter-individual differences were observed in pharmacokinetic parameters of resveratrol in humans (i.e. high coefficient of variations).[537, 538] This suggests variability in the nature and quantity of metabolites formed after oral administration.[538] Furthermore, enzymes that reconvert metabolites back to parent compounds (sulphatases and glucuronidases) are not ubiquitously secreted and thus may not wholly explain the biological activity observed despite low bioavailability. Although some metabolites have shown activity similar to their parent compounds, little or no biological effects of resveratrol glucuronide have also been reported.[305] Most human studies do not examine levels of resveratrol in tissues. It is possible that tissue levels of stilbenoids are higher than that detectable in plasma.

The dose used in this study (2.5 mg/kg/day) was based on previous studies that used similar doses of resveratrol in SHR. The human equivalent dose is 24.3 mg/day for a 60-kg human with

consideration of inter-species differences and body surface area. Although some clinical studies have used higher doses of resveratrol (1-5g/day),[539, 540] comparably low doses (10-30 mg/day) have also been employed in other human studies.[541, 542] The rationale for choosing such a low dose is that we are proposing a supplementary and preventative regimen that may be used alongside current therapy particularly in patients at risk for heart failure. Moreover, high dose resveratrol, particularly the micronized form, has been associated with renal toxicity in multiple myeloma patients.[543]

# CHAPTER 8

# CONCLUSION AND FUTURE DIRECTIONS

# 8.0 CHAPTER EIGHT: CONCLUSION AND FUTURE DIRECTIONS

Similar to resveratrol, gnetol and pterostilbene inhibited ET-1-induced hypertrophy in isolated neonatal rat ventricular cardiomyocytes. The mechanism of anti-hypertrophic effects of gnetol and pterostilbene were, at least in part, via activation of AMPK. The cardioprotective effect of stilbenoids *in vivo* extends beyond their potential anti-hypertrophic actions. Although neither resveratrol, gnetol nor pterostilbene inhibited cardiac hypertrophy in SHHF rat model, they improved diastolic function by preventing prolongation of IVRT. The apparent differences in bioavailabilities of the stilbenoids studied in this project did not seem to affect their respective bioactivities, since they all improved diastolic function to the same extent. Therefore, the low bioavailability of stilbenoids may not necessarily be a limitation to their biological activities, although possible inter-individual differences in the metabolism and distribution of stilbenoids might hinder extrapolation to humans.

This is the first report of the effects of resveratrol (and other stilbenoids) in the SHHF rat model. Since previous studies have shown that resveratrol prevents cardiac hypertrophy in SHR model, a similar comparative study of stilbenoids in SHR model may prove worthwhile. This way the effect of gnetol and pterostilbene on cardiac hypertrophy can be easily compared with that of resveratrol. The lack of effect of resveratrol on hypertrophy in SHHF rat indicates model-specific differences between genetic models of heart disease. Indeed, SHHF is a more complex model which superimposes hypertension over propensity to develop heart failure and thus may respond differently to stilbenoid treatment.

Directions for future pharmacokinetic studies should also put more emphasis on tissue bioavailability of the stilbenoids rather than only the plasma concentrations. It is possible that

higher concentrations of stilbenoids are achievable in the tissue. For instance, in correlation with the cardioprotective effects, determination of stilbenoid concentrations in the heart tissue might provide more valuable information.

Limitations of this study include the short period of study (8 weeks). A longer duration of study may possibly yield a greater effect on hypertrophy. Although lower doses were administered in this study, higher doses could result in a different outcome. Moreover, the animals in this study were 9 weeks old at the onset of the treatment. It is possible that slightly earlier intervention may impact the outcome of the study. An earlier onset of treatment may prevent hypertrophy before it develops in the early stage.

The role of MITF in the anti-hypertrophic effects of stilbenoids remains unclear. Results from this study support the existing notion that MITF plays a role in development of cardiomyocyte hypertrophy. Although stilbenoids prevented increase in MITF expression induced by ET-1, it cannot be concluded that stilbenoids inhibited hypertrophy via repression MITF expression. Further studies will be required to determine the role of MITF signalling in the stilbenoid effects. The fact that resveratrol increased MITF expression, in contrast to gnetol and pterostilbene, also warrants further study.

Overall, outcomes of this study indicate possible application of stilbenoids in cardioprotection in the heart failure setting. Although there was no effect on hypertrophy in the SHHF model, this study and other studies have shown improvement of cardiac function in the absence of anti-hypertrophic effects. Thus, the cardioprotective potential of stilbenoids should not be ignored. However, human studies will eventually be needed to support the cardioprotective claim. In future practice, stilbenoids may be recommended as supplements to existing therapy to improve outcomes in cardiovascular diseases.

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Select figures and texts in chapters 4 to 7 have been published in original article: Disparate Effects of Stilbenoid Polyphenols on Hypertrophic Cardiomyocytes In Vitro vs. in the Spontaneously Hypertensive Heart Failure Rat. *Molecules*. 2017;22. by Akinwumi BC, Raj P, Lee DI, Acosta C, Yu L, Thomas SM, Nagabhushanam K, Majeed M, Davies NM, Netticadan T and Anderson HD.
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