

Mechanistic Study on Cardiovascular Protective Effects of A  
Novel Danshensu Derivative

by

Guozhen Cui

Doctor of Philosophy in Biomedical Sciences

2013



Institute of Chinese Medical Sciences

University of Macau



Mechanistic Study on Cardiovascular Protective Effects of A  
Novel Danshensu Derivative

by

Guozhen Cui

SUPERVISOR: Prof. Simon Ming-Yuen Lee

CO-SUPERVISOR: Dr. Maggie Pui-Man Hoi

Doctor of Philosophy in Biomedical Sciences

2013

Institute of Chinese Medical Sciences

University of Macau



Author's right 2013 by  
Cui, Guozhen





## **Acknowledgements**

First and foremost, I would like to thank my supervisor, Prof. Simon Ming-Yuen Lee. Without his guidance, encouragement, enlightenment and tireless support, this thesis would not have been possible. The help he gave me during my study can never be repaid. Special thanks to my co-supervisor, Dr. Maggie Pui-Man Hoi, who gave valuable suggestions and kind assistance, more importantly, revision and proof reading of my manuscripts and thesis that made the bad days bearable. I would especially like to thank current and former members of Dr. Lee's lab, who tried to teach me everything they knew when I started the project.

I am eternally grateful to Prof. Yi Tao Wang for giving me such a good chance to do scientific work. Additional thanks to Shao Ping Li, Ying Zheng, Qing Wen Zhang for their suggestions and kind help during my PhD study.

I would like to thank the entities that funded this project. The current research was supported by grants from the Science and Technology Development Fund Macao SAR, China (Ref. No. 014/2011/A1).

Thanks to lab technicians, Miss. Sandy, Miss. Wing, Miss. Joanna, Ms. Leon for their hard work and patience to keep the lab running. I would like to thanks Ms. Nan

who maintains the zebrafish room running and the fish happy. For providing me with a good environment during my study, I am grateful to the members of administrative staff Hattie, Chloe and Ada.

Additional thanks to all ICMS colleagues for their help and discussions during the execution of this work in ICMS. They have made doing scientific work memorable. Thanks to Zai Jun Zhang, Lei Si wai, Shang Li, Cheong Meng Chong, Liang Wang, Guo Shen Wu, Hisa, Zhi Qiang Dong, Hai Tao Li, Lam In Kei as well as other classmates and friends who studied here for offering me their unlimited support.

In addition, I would especially like to thank a number of scientific collaborators: Prof. Yu Qiang Wang, Lu Chen Shan and other members from Jinan University for compound synthesis, suggestions and kind assistance in animal study during the past 4 years. They will give happy memories in doing scientific research and they encouraged me to do my best. Prof. Ivan Chu, Guo Hui Li and Dr. Ken Chan from the University of Hong Kong for their help in proteomics work and human cardiomyocytes inducing. Prof. Yi Fan Han and Wei Cui from the Hong Kong Polytechnic University for their kind help during my PhD study.

Last, but certainly not least, it is difficult to estimate my appreciation to my family. Although they have never quite understood what I did in lab, they always supported and encouraged me throughout my graduate career. Many thanks to you all!



---

**Abstract**

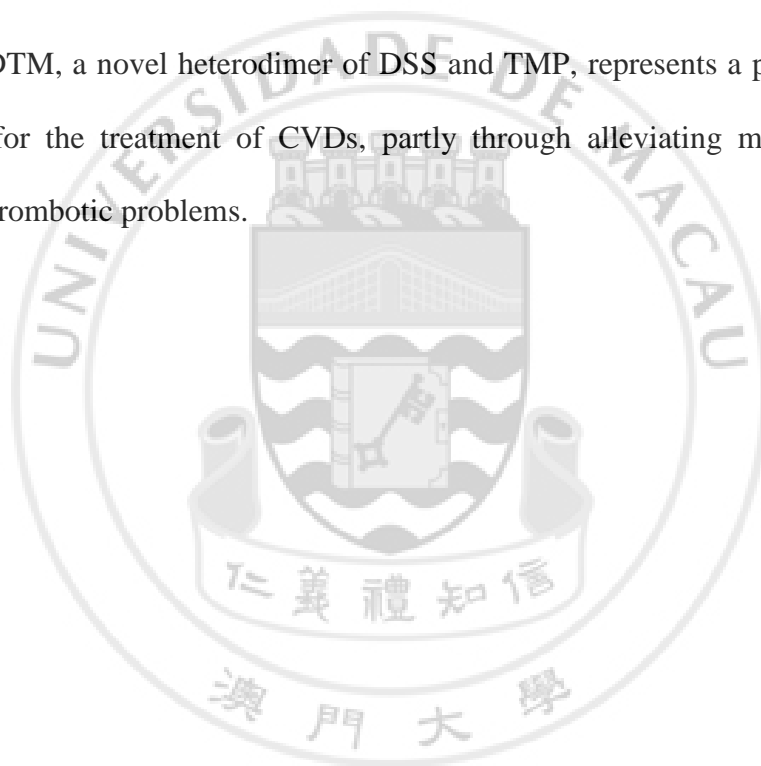
Cardiovascular disease (CVD) is the leading cause of global mortality and its prevalence is still increasing. Despite current drug treatments that can significantly improve the clinical outcomes in patients with CVD, adverse effects or no pharmacological responses exist in some cases. Hence, there remains an unmet need for developing novel pharmacological agents with improved efficacy and free of the side effects to prevent CVD.

Danshensu (DSS) and tetramethylpyrazine (TMP) are major active ingredients from danshen and chuanxiong, respectively. Previous studies indicated that additive synergistic effects may exist when danshen and chuanxiong are co-administered. In view of these results, we postulated that the beneficial effects of danshen and chuanxiong might be exploited by the combination of the pharmacophoric moieties of DSS and TMP. We had synthesized a novel compound named ADTM by modifying a heterodimer of DSS and TMP. The activities of ADTM were investigated in numerous experimental models of cardiovascular diseases.

ADTM was much more potent than its parent compounds, DSS and TMP alone as well as in combination, against *tert*-butylhydroperoxide-induced cell injury in H9c2 cardiomyoblasts. ADTM treatment significantly alleviated myocardial infarction in a rat model of myocardial ischemia. Moreover, the PI3K/Akt and Nrf2 pathways were found to be involved in the cardioprotective effect of ADTM both *in vitro* and *in vivo*. In addition, ADTM demonstrated a more potent and broader spectrum of anti-platelet aggregation activity than its parent compounds in *in vitro* and *in vivo*. In order to identify the protein interacting targets of ADTM, pull-down proteins bound with

biotinylated modified ADTM chemical structure were analyzed by LC-MS/MS. We had identified and confirmed protein isomerase disulfide (PDI) family members, particular ERp57, as novel interacting targets of ADTM. The mechanisms underlying the anti-platelet aggregation effect of ADTM, at least in part, are mediated by the inhibiting the activity of ERp57 as well as activating vasodilator-stimulated phosphoprotein (VASP) and heme oxygenase-1 (HO-1), leading to suppress platelets surface expression of  $\alpha$ IIb $\beta$ 3 and P-selectin.

Collectively, ADTM, a novel heterodimer of DSS and TMP, represents a promising lead candidate for the treatment of CVDs, partly through alleviating myocardial infarction and thrombotic problems.



### **Declaration**

I declare that the thesis here submitted is original except for the source materials explicitly acknowledged and that this thesis as a whole, or any part of this thesis has not been previously submitted for the same degree or for a different degree.

I also acknowledge that I have read and understood the Rules on Handling Student Academic Dishonesty and the Regulations of the Student Discipline of the University of Macau.

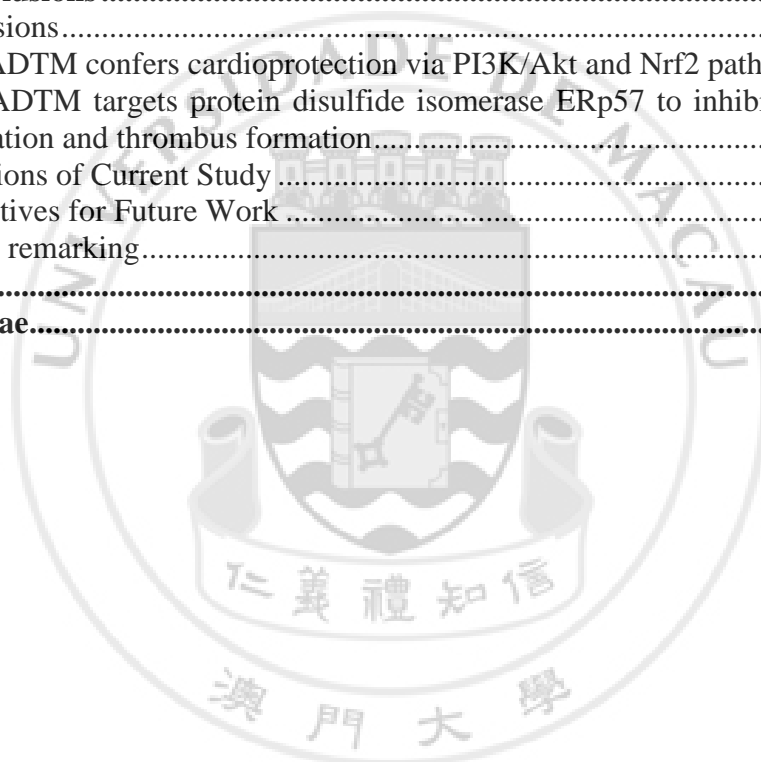


## Table of Contents

<b>Acknowledgements</b> .....	<b>i</b>
<b>Abstract</b> .....	<b>iii</b>
<b>Declaration</b> .....	<b>v</b>
<b>List of Tables and Figures</b> .....	<b>ix</b>
<b>List of Abbreviations</b> .....	<b>x</b>
<b>Chapter 1 Introduction</b> .....	<b>1</b>
1.1. General Background .....	1
1.1.1. Epidemic status, types, mechanisms and causes of cardiovascular diseases .....	1
1.1.2. Ischemic heart disease and current treatment drugs .....	2
1.1.3. Platelets and CVD .....	3
1.1.4. Current antiplatelet therapy .....	3
1.1.4.1. Current antiplatelet drugs .....	4
1.1.4.2. Chinese herb for promoting blood circulation and removing blood stasis .....	6
1.1.5. Drug targets .....	6
1.1.6. Brief summary .....	7
1.2. Specific Background.....	8
1.2.1. Danshensu, tetramethylpyrazine and its derivative .....	8
1.2.1.1. Danshensu .....	8
1.2.1.2. Tetramethylpyrazine .....	11
1.2.1.3. DSS and TMP derivative .....	14
1.2.2. Discovery of drug targets by chemical proteomics approach.....	14
1.2.3. Protein disulfide isomerase family and disease .....	15
1.3. Research Goals and Objectives.....	18
1.3.1. General Goals and Objectives .....	18
1.3.2. Specific Goals and Objectives .....	18
1.4. Research Methodology and Design .....	19
1.5. Potential Contributions .....	21
1.6. Organization of the Thesis .....	22
1.7. Statement of Originality.....	23
<b>Chapter 2 ADTM confers protective effects against oxidative stress-induced cell injury in H9c2 cells and myocardial injury in rat</b> .....	<b>25</b>
2.1. Introduction.....	25
2.2. Materials and methods .....	26
2.2.1. Materials .....	26
2.2.2. Cell culture .....	27
2.2.3. Cell treatment and MTT assay.....	27
2.2.4. Lactate dehydrogenase (LDH) assay .....	28
2.2.5. Fluorescein isothiocyanate (FITC)-labeled Annexin V-based apoptosis assay .....	28
2.2.6. Measurement of mitochondrial membrane potential ( $\Delta\psi_m$ ).....	28
2.2.7. Detection of Intracellular Reactive Oxygen Species (ROS) .....	29
2.2.8. Immunostaining assay .....	29
2.2.9. Nrf2 transcriptional activity.....	29
2.2.10. Preparation of whole cell, cytosolic and nuclear extracts .....	30
2.2.11. Nrf2 siRNA transfection.....	31
2.2.12. Immunoprecipitation .....	31

2.2.13. Western blot analysis.....	31
2.2.14. Model of acute myocardial ischemia, drug treatment and determination of the infarct size.....	32
2.2.15. Statistical Analysis.....	33
2.3. Results.....	33
2.3.1. Protective effects of ADTM on <i>t</i> -BHP-induced cell injury in H9c2 cardiomyoblast cells.....	33
2.3.2. ADTM inhibited apoptosis induced by <i>t</i> -BHP in H9c2 cells.....	35
2.3.3. ADTM pretreatment attenuated <i>t</i> -BHP-induced decrease in mitochondrial activity.....	36
2.3.4. Oxidant scavenging property of ADTM on <i>t</i> -BHP-induced ROS generation.....	37
2.3.5. Effects of ADTM on PI3K/Akt signaling.....	38
2.3.6. Effects of ADTM on the activity of PI3K, Akt and GSK-3 $\beta$ in H9c2 cells against <i>t</i> -BHP-induced cell injury.....	39
2.3.7. ADTM activates the Nrf2/Keap1 pathway.....	41
2.3.8. Nrf2-siRNA transfection abolished the cytoprotection of ADTM in <i>t</i> -BHP-treated H9c2 cells.....	43
2.3.9. ADTM activated the Nrf2/HO-1 signaling pathway which was mediated by PI3K/Akt.....	44
2.3.10. Protective effects of ADTM after acute myocardial ischemia in rat.....	45
2.3.11. ADTM activated the PI3K/Akt and Nrf2/HO-1 pathways <i>in vivo</i> ..	47
2.4. Discussion.....	48
<b>Chapter 3 ADTM inhibits platelet aggregation and thrombus formation.....</b>	<b>52</b>
3.1. Introduction.....	52
3.2. Materials and methods.....	54
3.2.1. Materials.....	54
3.2.2. Preparation of platelet-rich plasma.....	55
3.2.3. Protein preparation from platelets.....	55
3.2.4. Western blot analysis.....	55
3.2.5. Platelet aggregation <i>in vitro</i> .....	56
3.2.6. Determination of platelet activation by flow cytometry.....	56
3.2.7. Rat platelet aggregation-induced by ADP <i>in vivo</i> .....	57
3.2.8. FeCl <sub>3</sub> -induced inferior vena cava thrombosis in rat.....	57
3.2.9. Evaluation of plasma 6-Keto-PGF <sub>1<math>\alpha</math></sub> levels in rat.....	58
3.2.10. Statistical analysis.....	58
3.3. Results.....	59
3.3.1. ADTM inhibits platelet aggregation-induced by ADP, AA and thrombin.....	59
3.3.2. ADTM inhibits activation of $\alpha$ IIB $\beta$ 3.....	61
3.3.3. ADTM inhibits ADP-induced P-selectin expression.....	61
3.3.4. ADTM inhibits platelet aggregation-induced by ADP <i>in vivo</i> .....	62
3.3.5. ADTM inhibits thrombus formation <i>in vivo</i> .....	64
3.3.6. ADTM reverses the decrease in 6-Keto-PGF <sub>1<math>\alpha</math></sub> level-induced by FeCl <sub>3</sub> <i>in vivo</i> .....	64
3.3.7. ADTM enhances VASP phosphorylation and HO-1 protein levels..	66
3.4. Discussion.....	67
<b>Chapter 4 Identification of a primary target of ADTM.....</b>	<b>69</b>
4.1. Introduction.....	69
4.2. Materials and methods.....	70

4.2.1. Materials .....	70
4.2.2. Preparation of platelet-rich plasma.....	70
4.2.3. Protein preparation from platelets .....	70
4.2.4. NeutrAvidin Agarose Resin pull-down with BDB.....	71
4.2.5. Nano LC-MS/MS analysis.....	71
4.2.6. Database Search and Data Analysis .....	72
4.2.7. Western blot analysis.....	72
4.3. Results.....	73
4.3.1. Identification of ADTM binding with PDI protein family members by chemical proteomics approach .....	73
4.3.2. Validation of binding between ADTM and PDI protein family by Western blot analysis.....	75
4.3.3. ADTM inhibits redox activities of PDI family.....	77
4.4. Discussion.....	78
<b>Chapter 5 Conclusions .....</b>	<b>81</b>
5.1. Conclusions.....	81
5.1.1. ADTM confers cardioprotection via PI3K/Akt and Nrf2 pathways..	83
5.1.2. ADTM targets protein disulfide isomerase ERp57 to inhibit platelet aggregation and thrombus formation.....	83
5.2. Limitations of Current Study .....	83
5.3. Perspectives for Future Work .....	84
5.4. Closing remarking.....	85
<b>References .....</b>	<b>87</b>
<b>Curriculum Vitae.....</b>	<b>97</b>



## List of Tables and Figures

Fig. 1.1. Platelet function and targets of antiplatelet pharmacological agents.....	4
Fig. 1.2. Chemical structure of DSS .....	8
Fig. 1.3. Chemical structure of TMP .....	11
Fig. 1.4. Research methodology and design (A).....	19
Fig. 1.5. Research methodology and design (B).....	20
Fig. 2.1. Evaluation of cytoprotective effects for DSS, TMP and ADTM. ....	34
Fig. 2.2. Effects of ADTM on <i>t</i> -BHP-induced apoptosis in H9c2 cells.....	35
Fig. 2.3. Effects of ADTM on <i>t</i> -BHP-induced dissipation of mitochondrial membrane potential ( $\Delta\psi_m$ ) and intracellular oxidant level in H9c2 cells. ....	37
Fig. 2.4. ADTM significantly reduced ROS level induced by <i>t</i> -BHP.....	38
Fig. 2.5. Involvement of the PI3K/Akt pathway in cardioprotection by ADTM in H9c2 cells.....	39
Fig. 2.6. ADTM activates PI3K/Akt pathway. ....	40
Fig. 2.7. ADTM increased protein expression of Nrf2 and HO-1, but decreased Keap1 protein in a time-dependent manner in H9c2 cells. ....	41
Fig. 2.8. ADTM confers cardioprotection through the activation of Keap1/Nrf2 signaling in H9c2 cells. ....	42
Fig. 2.9. ADTM disrupted the Keap1-Nrf2 complex.....	43
Fig. 2.10. Nrf2 siRNA transfection blocked the cardioprotection by ADTM. ....	44
Fig. 2.11. ADTM-induced Nrf2 signaling was dependent on PI3K/Akt. ....	45
Fig. 2.12. Cardioprotective effect of ADTM in rats. ....	46
Fig. 2.13. Involvement of PI3K/Akt and Nrf2 pathways in cardioprotection conferred by ADTM in rat heart ischemia model. ....	48
Fig. 3.1. The inhibitory effects of ADTM on ADP, AA and thrombin-induced platelet aggregation <i>in vitro</i> . ....	60
Fig. 3.2. ADTM inhibits surface expression of activated $\alpha$ IIb $\beta$ 3 and P-selectin on platelets. ....	62
Fig. 3.3. ADTM inhibites platelet aggregation-induced by ADP in a concentration dependent manner <i>in vivo</i> .....	63
Fig. 3.4. Anti-thrombotic effect of ADTM on FeCl <sub>3</sub> -induced thrombosis model in rat. ....	65
Fig. 3.5. Effects of ADTM on VASP phosphorylation and HO-1 protein expression. ....	66
Fig. 4.1. Chemical structures of DSS, TMP, ADTM, and BDB.....	74
Fig. 4.2. Schematic diagram for the target identification by a chemical proteomics approach. ....	74
Fig. 4.3. Target validation and the inhibition of the PDI members by ADTM.....	76
Fig. 4.4. Proposed mechanism of anti-platelet of ADTM.....	77
Fig. 5.1. Proposed mechanisms of beneficial effects conferred by ADTM on CVDs. ....	82
Table 1.1. Current main FDA-approved antiplatelet drugs.....	5
Table 1.2. Summary of the main biological activities of DSS.....	9
Table 1.3. Summary of the main biological effects of TMP reported from 2012 to 2013.....	12
Table 4.1. Proteins identified by pull down and mass spectrometry .....	75

**List of Abbreviations**

AA	Arachidonate
ADP	Adenosine diphosphate
CVD	Cardiovascular disease
DSS	Danshensu
ER	Endoplasmic reticulum
ERp	Endoplasmic reticulum protein
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HO-1	Heme oxygenase 1
IHD	Ischemic heart disease
IP	Immunoprecipitation
LDH	Lactate dehydrogenase
MS	Mass spectrometry
Nrf2	Nuclear factor erythroid 2-related factor 2
PDI	protein disulfide isomerase
PI3K	Phosphoinositide 3-Kinase
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
ROS	Reactive oxygen species
siRNA	Small interfering RNA
<i>t</i> -BHP	<i>tert</i> -Butylhydroperoxide
TMP	Tetramethylpyrazine
VASP	Vasodilator stimulated phosphoprotein