The discovery of novel ROCK inhibitors with anti-angiogenesis activity

By

Yang Binrui

Master of Science

2012

Institute of Chinese Medical Sciences

University of Macau

University of Macau

The discovery of novel ROCK inhibitors with anti-angiogenesis activity

By

Yang Binrui

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Chinese Medical Science

Master of Science

Institute of Chinese Medical Sciences

University of Macau

2012

Approved by

_____

Supervisor

_____

_____

_____

Date

_____

_____

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at the University of Macau, I agree that the Library and the Institute of Chinese Medical Sciences shall make its copies freely available for inspection. However, reproduction of this thesis for any purposes or by any means shall not be allowed without my written permission. Authorization is sought by contacting the author at

Address:

Telephone: 83974880 / 62161490

Fax:

E-mail: yangbr.cpu@gmail.com

Signature _____

Date _____

UNIVERSIDADE DE MACAU
仁義禮知信
澳門大學

澳門大學碩士學位論文

新结构 **Rho** 激酶抑製劑的发现及其抗血管新生作用

研究生姓名： 楊彬睿

導　　師： 李銘源

專　　業： 中藥學

日　　期： **2012.7**

澳門大學中華醫藥研究院

University of Macau

Abstract

The discovery of novel ROCK inhibitors with anti-angiogenesis activity

by Yang Binrui

Thesis Supervisor: Dr. Simon Ming Yuen Lee

Chinese Medicinal Science

Angiogenesis refers to the formation of new blood vessels from preexisting vasculature, and is essential in a series of physiologic processes such as embryonic development, wound healing and female reproductive cycle. Excessive or inefficiency angiogenesis may lead to pathologic response and diseases such as rheumatoid arthritis, diabetic retinopathy as well as tumor growth and metastasis.

Rho kinase is the major effector of GTPase Rho and plays an important role in regulation of rearrangement of cytoskeleton underlying several biological processes such as cell proliferation and migration as well as muscle contraction. The over activation of ROCK was found to associate with some cardiovascular diseases and thus the inhibition of ROCK became a potential therapy target of these diseases. Fasudil and Y-26732 are some examples of ROCK inhibitors which had been successfully developed into clinical stages. Computational virtual screening coupled with high-throughput assays are viable approaches to the discovery of ROCK inhibitor candidates. In present study, ten ROCK inhibitor candidates had been identified and further determined for their inhibitory effects on proliferation and

migration of human umbilical vein endothelial cells (HUVECs) as well as blood vessel formation of zebrafish. Three of the compounds, namely analogs 2, 4 and 7, were found to exert significant anti-angiogenesis effect on intersegmental vessel (ISV) formation of zebrafish. Since angiogenesis involves multiple biological processes such as proliferation, migration, invasion and differentiation of endothelial cell, we further investigated the differential effects of analogs 2, 4 and 7 on proliferation and migration assays. The results revealed that analog 7 exerted higher potency than analogs 2 and 4 on inhibiting HUVECs proliferation. By contrary, only analog 2 but not analog 4 and 7 suppressed HUVECs migration. These results suggest that different mechanisms of actions may be involved in their underlying anti-angiogenesis activities.

In short, the present study, for the first time, identified three novel ROCK inhibitors and demonstrated their anti-angiogenic effects on HUVEC and zebrafish. These evidences supporting that these three compounds are potential therapeutic agents for vascular dysfunction with excessive angiogenesis.

KEY WORDS: ROCK inhibitor，anti-angiogenesis, zebrafish, HUVECs, proliferation, migration

摘要

血管新生是在已有的血管組織網絡中形成新血管的過程。血管新生涉及胚胎發育，創傷癒合和女性生理週期等多個生理過程，但過多或不足的血管新生會導致一些病理變化甚至發展為疾病，例如類風濕關節炎，糖尿病視網膜病變，腫瘤的形成和轉移。

Rho 激酶是 Rho 激活后主要的效應器，能够調節細胞骨架的重構，與細胞遷移、黏附、肌細胞收縮等多項生理功能相關。有研究報導在一些心血管疾病中發現了 Rho 激酶的高度激活，提示了 Rho 激酶的抑制是此類疾病可能的治療途徑。Y-26732 和法舒地爾是臨床前研究和臨床應用最常用 Rho 激酶抑製劑。計算機擬合和酶反應檢測在 Rho 激酶抑製劑的篩選中應用廣泛，一些有潛力的 Rho 激酶抑製劑也相继被发现。

本課題研究篩選了十種化合物的對 Rho 激酶抑製活性，并研究了這十種化合物對人臍靜脈內皮細胞增值、遷移和對斑馬魚節間血管新生的影響。其中三種化合物 2, 4 和 7 表現出較強的 Rho 激酶抑製劑活性且具有抑制斑馬魚節間血管的形成的活性，但是這三種化合物對人臍靜脈內皮細胞的增值和遷移的影響不同。化合物 7 表現出顯著的抗增殖作用而化合物 2 表現出顯著的抗遷移作用，這提示了他們可能潛在的不同的作用機理。

綜上所述，此次研究首次鑒定了化合物 2, 4 和 7 的 Rho 激酶抑製劑活性和在斑馬魚和人臍靜脈內皮細胞的抗血管新生活性，對與以後利用其作為治療血管功能紊亂類疾病中的血管新生過剩癥狀的提供了依據。

關鍵字： Rho 激酶抑製劑；抗血管新生；斑馬魚；人臍靜脈內皮細胞；細胞遷移；細胞增殖

ACKNOWLEDGEMENTS

During my master study in Institute of Chinese Medical Sciences (ICMS) these two years, I have learnt a lot either in researches or other aspects. Here, especially, I would like to owe my deepest gratitude to my supervisor Professor Simon Ming-Yuan Lee for his advice and encouragement throughout my study. And also I would like to thank Professor Yitao Wang for giving me this opportunity to study here and provided valuable resources. I would also like to express my grateful to all teachers in ICMS for the counsels throughout my studies. I benefited a lot from their lectures and counsels, especially the diligent attitude and rigorous spirit for scientific research.

Many thanks to all members of ICMS for all the help in these years. My sincere appreciation to Sandy Lao, Leung Chi Weng and Joanna, they taught me valuable experimental skills and never expressed boredom for help me in my research. Special thanks to Dr. Zhang Zaijun, Cui Guozhen, Li Shang, Vincent, Xu Daping, Ling ling, Ginny, Zhang Zherui, Yuan Shuai, Lorrisa for research and technical support. Also thank to all of the staff of ICMS, Hattie U, Ada Wong, Leon Lai, Kio and etc.

Many thanks to my laboratory colleagues and friends, for their warm concern and giving me the great memory of the happiness during these years. And I sincerely wish a bright future and happiness for everyone in ICMS.

Finally, I would like to express my thanks to the my family and my boyfriend for their encouragement and love without end.

TABLE OF CONTENT

FIGURES LIST i
ABBREVIATIONS iii
Chapter 1 General Introduction 1
1.1 Angiogenesis 1
1.1.1 Introduction of angiogenesis 1
1.1.2 Regulation of angiogenesis 3
1.1.3 Angiogenesis and diseases 8
1.1.4 Preclinical models for discovering anti-angiogenic agents 11
1.1.4.1 Endothelial cell proliferation assay 11
1.1.4.2 Endothelial cell migration assay 12
1.1.4.3 Endothelial cell tube formation assay 12
1.1.4.4 The rat and mouse aortic ring assays 13
1.1.4.5 The Matrigel plug and sponges or polymer implant assay 14
1.1.4.6 The chick choriallantoic membranes(CAM) assay 15
1.1.4.7 The corneal pocket assay 15
1.1.4.8 Zebrafish assay 16
1.2 Rho associated protein kinase (ROCK) 19
1.2.1 General introduction of Rho associate protein kinase(ROCK) 19
1.2.2 ROCK regulation 20
1.2.3 ROCK and physiopathology 22
1.2.4 ROCK inhibitors 24
Chapter 2 Materials and Methods 27
2.1 Ethics Statement 27
2.2 Chemicals, regents, cell line and animals 27
2 3 Enzymatic assay of ROCK inhibitors 28
2.4 Zebrafish maintenance and embryo handling 28
2.5 Survival and Toxicity of selected ROCK inhibitors on WT zebrafish 29
2.6 Anti-angiogenic morphological observation on Tg(fli1:EGFP)

zebrafish 29
2.7 HUVECs culture 29
2.8 Cell proliferation assay 30
2.9 Cell migration assay 30
2.10 Statistic 30
Chapter 3 Results 32
3.1 Schematic illustration of the study design 32
3.2 Effects of enzymatic assay 33
3.3 Toxicity of ROCK inhibitors on wil dtype (WT) zebrafish 34
3.4 Effect of ten ROCK inhibitors on the vasculature morphology of Tg(fli1:EGFP) zebrafish 36
3.5 Anti-angiogenic activity of analog 2, 4, and 7 on Tg(fli1:EGFP) zebrafish 39
3.6 Effect of selected ROCK inhibitors on HUVECs proliferation 42
3.7 Effect of selected ROCK inhibitors on HUVECs migration 44
Chapter 4 Discussion and conclusion 46
4.1 Discussion 46
4.2 Conclusion 50
Bibliography 51