UNIVERSITI PUTRA MALAYSIA

ANTIFIBROTIC EFFECT OF TRANSFORMING GROWTH FACTOR BETA 1 INHIBITOR EXTRACT FROM STREPTOMYCES SP. STRAIN H6552 ON HUMAN HEPATIC STELLATE CELLS

LIM CHOOI LING

FPSK(p) 2010 1

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By

LIM CHOOI LING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2010

DEDICATION

~ This thesis is especially dedicated to my dearest husband, Ker Yang, and father, Lim Loong Fatt; one who has dedicated his life to medicine and patient care, and the other to Science education.

A short history of medicine

"Doctor, I have an earache…"

Doctor's reply:

2000 B.C. – "Here, eat this root"

1000 B.C. – "That root is heathen, say this prayer"

1850 A.D. – "That prayer is superstition, drink this potion"

1940 A.D. – "That potion is snake oil, swallow this pill"

1985 A.D. – "That pill is ineffective, take this antibiotic"

2000 A.D. – "That antibiotic is artificial. Here, eat this root."

We have inevitably come full circle.

~ Author unknown

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair : Professor Seow Heng Fong, PhD

Faculty : Faculty of Medicine and Health Sciences

Liver fibrosis is a result of the body's natural wound healing response, but excessive scarring leads to significant morbidity and mortality. Transforming growth factor-beta1 (TGF-β1) inhibitors that hinder the fibrotic mechanism are currently being developed. However, an effective anti-fibrotic drug remains elusive, and *in vitro* anti-fibrotic studies using hepatic stellate cells (HSCs) are often complicated by the dynamic plasticity of these cells which become spontaneously activated in culture. In this study, we aimed to assess the quiescing effect of seeding LX-2 human HSC line on Matrigel-coated culture plates, and evaluate the anti-fibrotic activity of soil-derived *Streptomyces (S.)* sp. H6552 extract and/or active fraction, and SB 431542 (a commercial TGF-β receptor inhibitor) on LX-2 cells.

In HSC culture studies, LX-2 cells were seeded either on non-coated or Matrigel-coated culture plates and subjected to fibrotic marker analyses, Oil Red O staining,

and phase contrast microscopy. In the next chapter, *S.* sp. H6552 was cultured in mannitol-peptone medium and its metabolites were isolated via a 'shake-flask' method followed by acetone extraction, HPLC analysis and fractionation of crude H6552 extract. A bioassay-guided screening selection yielded the potential bioactive fraction (F3). Viability tests (MTT assay) were performed to evaluate the cytotoxicity of the crude extract. LX-2 cells were then treated with either the extract, F3 or SB 431542 with or without 8 to 10 ng/mL TGF- β1 induction, followed by assays for anti-fibrotic activity. Proliferation of cells were assessed via ^{3}H-thymidine incorporation, mitochondrial stress was evaluated by MitoTracker Red® fluorescence staining, and cytoplasmic lipid accumulation analyses for quiescence determination was performed via Oil Red O staining. TGF-β1 inhibitory activity was evaluated by Smad reporter and IgA promoter luciferase assays, while expression of fibrotic markers were analysed via Real-Time PCR, immunoblotting, and immunocytochemistry.

A progressively activated morphology was observed in LX-2 cells with prolonged culture on plastic, but this phenomenon was inhibited on Matrigel attachment substrate whereby an adipocytic, quiescent phenotype was conserved with concurrent reduction in TGF-β1-induced alpha-smooth muscle actin (α-sma) protein expression. *S.* sp. H6552 extract was found to be non-cytotoxic but exerted strong anti-proliferative activity from 1 mg/mL compared to untreated control ($p<0.01$), while the influence of F3 on proliferation was insignificant. Mitochondrial staining showed a possible antioxidative effect of 2 mg/mL H6552 crude extract on LX-2 cells, while 100 µg/mL F3 induced a quiescent, adipocytic phenotype in 73.85 ± 2.50% of treated

cells ($p<0.05$). Smad3 reporter activity was inhibited by 50% after 2 mg/mL crude extract treatment compared to TGF-β1-induced cells ($p<0.01$). TGF-β1-stimulated α-sma mRNA expression was attenuated by crude extract (from 0.125 mg/mL) and F3 (25 µg/mL) treatment, and protein-level α-sma inhibition was also apparent ($p<0.05$). SB 431542 (25 µM) inhibited proliferation, TGF-β1 (8 ng/mL)-induced Smad3 activation via abrogation of CAGA-luc Smad reporter activity, and α-sma protein and mRNA expression in LX-2 cells ($p<0.01$). In conclusion, we demonstrated that Matrigel may be a useful culture substrate to maintain LX-2 quiescence in *in vitro* studies, and *S.* sp. H6552 extract, F3, and SB 431542 exert anti-fibrotic activity towards human HSCs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Kedoktoran

KESAN ANTIFIBROTIK EKSTRAK PERENCAT 'TRANSFORMING GROWTH FACTOR BETA 1' DARIPADA *STREPTOMYCES* SP. STRAIN H6552 TERHADAP SEL HATI 'STELLATE' MANUSIA

Oleh

LIM CHOOI LING

Januari 2010

Pengerusi : Profesor Seow Heng Fong, PhD

Fakulti : Fakulti Perubatan dan Sains Kesihatan

Fibrosis hepar merupakan tindakbalas semulajadi pemulihan badan terhadap kecederaan, tetapi penghasilan parut yang terlampau menyebabkan keparahan yang signifikan dan juga maut. Penghasilan perencat-perencat 'Transforming growth factor-beta1' (TGF-β1) yang menyahaktifkan mekanisme fibrosis kini dalam proses perkembangan. Walaubagaimanapun, sehingga kini tiada penawar yang efektif untuk fibrosis hepar. Kajian anti-fibrosis *in vitro* menggunakan sel hati 'stellate' (SHS) turut dimudaratkan oleh sifat sel-sel tersebut yang dinamik dan cenderung menjadi aktif dalam kultur. Kajian ini bertujuan untuk menilai kesan penyahaktifan SHS manusia (LX-2) apabila dikultur di atas lapisan Matrigel, dan juga untuk mengkaji aktiviti anti-fibrotik ekstrak dan/atau pecahan bioaktif mikrob *Streptomyces* (*S.*) sp. H6552, dan SB 431542 (perencat reseptor TGF-β komersil) terhadap sel LX-2.

Dalam kajian pertumbuhan SHS, sel-sel LX-2 dikultur di atas plastik atau permukaan yang berlapikkan Matrigel, serta diikuti dengan analisis penanda fibrotik, pewarnaan ’Oil Red O’, dan kajian mikroskopi fasa kontras. Bab yang seterusnya melibatkan kultur *S.* sp. H6552 dalam medium ‘mannitol-peptone’, perolehan metabolit sekunder melalui kaedah ’kelalang-goncang’, dan diikuti dengan ekstraksi aseton, analisis HPLC, dan penghuraian ekstrak mentah kepada pecahan-pecahannya. Pecahan bioaktif (F3) diperolehi melalui penyaringan berasaskan ’bioassay’. Ujian viabiliti (ujian MTT) dijalankan untuk menilai kesan sitotoksik ekstrak mentah. Seterusnya, sel-sel LX-2 didedahkan kepada ekstrak mentah, F3, ataupun SB 431542, dengan atau tanpa kehadiran 8 sehingga 10 ng/mL TGF-β1, serta diikuti oleh kajian aktiviti anti-fibrotik. Perkembangan pertumbuhan sel-sel, tekanan oksidatif mitokondria, dan penghimpunan lemak kandungan sitoplasma masing-masing dikaji dengan menggunakan ujian inkorporasi ^{3}H-thymidine, pewarnaan floresen MitoTracker Red®, dan pewarnaan ’Oil Red O’. Aktiviti perencatan TGF-β1 dikaji melalui ujian ’luciferase’ pelapor Smad dan promoter IgA, manakala ekspresi penanda fibrotik dianalisis dengan menggunakan ’Real-Time PCR’, ’imunoblot’, dan pewarnaan sel imuno-kimia.

Sel-sel LX-2 menunjukkan morfologi fenotaip aktif setelah dikultur beberapa hari di atas permukaan plastik. Akan tetapi, dengan penggunaan Matrigel, sel-sel diperhatikan mempunyai ciri sel lemak dan pasif dengan ekspresi protein aktin otot licin alfa (α-sma) yang berkurangan. Esktrak *S.* sp. H6552 didapati tidak sitotoksik tetapi mengakibatkan perencatan pertumbuhan signifikan pada dos 1 mg/mL jika dibandingkan dengan kumpulan kawalan ($p<0.01$), manakala F3 tidak mempengaruhi

pertumbuhan sel. Pewarnaan mitokondria menampilkan aktiviti antioksidan yang mungkin oleh 2 mg/mL ekstrak H6552 terhadap sel-sel LX-2, manakala pendedahan kepada 100 µg/mL F3 menyebabkan 73.85 ± 2.50% daripada sel-sel terlibat memperolehi fenotaip pasif dan berlemak ($p<0.05$). Aktiviti pelapor Smad3 direncatkan sebanyak 50% selepas pendedahan kepada 2 mg/mL ekstrak H6552 berbanding dengan sel-sel yang diujah dengan TGF-β1 ($p<0.01$). Paras ekspresi gen α-sma yang diujah dengan TGF-β1 dikurangkan dengan rawatan ekstrak dan F3 sebanyak 0.125 mg/mL dan 25 µg/mL masing-masing, manakala perencatan protein α-sma juga jelas diperhatikan ($p<0.05$). SB 431542 (25 µM) membantutkan pertumbuhan populasi sel, merencatkan lintasan Smad3 melalui pelapor CAGA-luc Smad yang diaktifkan oleh 8 ng/mL TGF-β1, serta mengurangkan ekspresi protein dan gen α-sma dalam sel LX-2. ($p<0.01$). Kesimpulannya, Matrigel mungkin amat berguna sebagai substrat pertumbuhan untuk mengekalkan keadaan pasif LX-2, dan ekstrak *S.* sp. H6552, F3, serta SB 431542 berupaya merencatkan aktiviti fibrotik dalam SHS manusia.

ACKNOWLEDGEMENTS

I would like to convey my heartfelt appreciation to several individuals who helped make this project and thesis a success. My deepest gratitude goes to Prof. Dr. Seow Heng Fong, who guided me from the conception of ideas and materials through to the completion of the final written draft. Her patience and unconditional support were essential elements of this work. My co-supervisors, Dr. Maha bt. Abdullah, Dr. Sharmili Vidyadaran, and Prof. Dr. Ho Coy Choke (retired) deserve special mention for their helpful advice, assistance and review of my work.

My sincere gratitude goes to Yip Wai Kien, Kak Masriana, Mandy Leong, Leslie, Choo and Jap Meng for being such patient seniors from whom I learnt so much. I am truly indebted to Mr. Anthonysamy, who was an immense help in the technicalities of laboratory maintenance, and the administrative personnel of Aishah, Kak Zura and Marsitah, who never tires of keeping our paperwork running smoothly. My labmates, especially Rhun Yian, were always there for me to share in the happiness and disappointments of scientific endeavours.

The team of researchers in the Liver Lab of Mount Sinai School of Medicine, New York, was a catalyst to the progress of this project. Prof. Dr. Scott Friedman was an exceptional scientist and tutor - never short of new ideas, wisdom, and concern for his team. My sincere thanks also go to Johnny Loke, who was my guide and teacher, Dr. Feng and Dr. Guo from China, from whom I learnt technical skills and perseverance, Dr. Mirko Tarocchi, and the entire group of outstanding researchers who welcomed me as family throughout the long months of attachment. To Dr. Khor Tin Oo and family who took care of me during the trying times, I thank you.

Finally, but certainly not the least, I would like to thank my understanding husband and godmother as well as my family who were my pillars of strength and source of love and encouragement. My apologies and sincere gratitude goes to those whom I failed to mention but contributed to this project in any manner.

I certify that an Examination Committee has met on 5th January 2010 to conduct the final examination of **Lim Chooi Ling** on her Doctor of Philosophy (PhD) thesis entitled **"Anti-fibrotic Effect of Transforming Growth Factor Beta 1 (TGF-Beta1) Inhibitor Extract from *Streptomyces* Sp. Strain H6552 on Human Hepatic Stellate Cells**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 17 March 2010

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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LIM CHOOI LING

Date:

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