



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

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H6552 ON HUMAN HEPATIC STELLATE CELLS**

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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January 2010

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- β 1) 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- β 1 induction, followed by assays for anti-fibrotic activity. Proliferation of cells were assessed via ^3H -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- β 1 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- β 1-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 $\mu\text{g/mL}$ F3 induced a quiescent, adipocytic phenotype in $73.85 \pm 2.50\%$ of treated

cells ($p < 0.05$). Smad3 reporter activity was inhibited by 50% after 2 mg/mL crude extract treatment compared to TGF- β 1-induced cells ($p < 0.01$). TGF- β 1-stimulated α -sma mRNA expression was attenuated by crude extract (from 0.125 mg/mL) and F3 (25 μ g/mL) treatment, and protein-level α -sma inhibition was also apparent ($p < 0.05$). SB 431542 (25 μ M) inhibited proliferation, TGF- β 1 (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

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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- β 1) 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- β 1, 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 ^3H -thymidine, pewarnaan floresen MitoTracker Red®, dan pewarnaan 'Oil Red O'. Aktiviti perencatan TGF- β 1 dikaji melalui ujian 'luciferase' pelapor Smad dan promoter IgA, manakala ekspresi penanda fibrotik dianalisis dengan menggunakan 'Real-Time PCR', 'imunoblot', dan pewarnaan sel immuno-kimia.

Sel-sel LX-2 menunjukkan morfologi fenotip 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. Ekstrak *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 $73.85 \pm 2.50\%$ 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.

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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.

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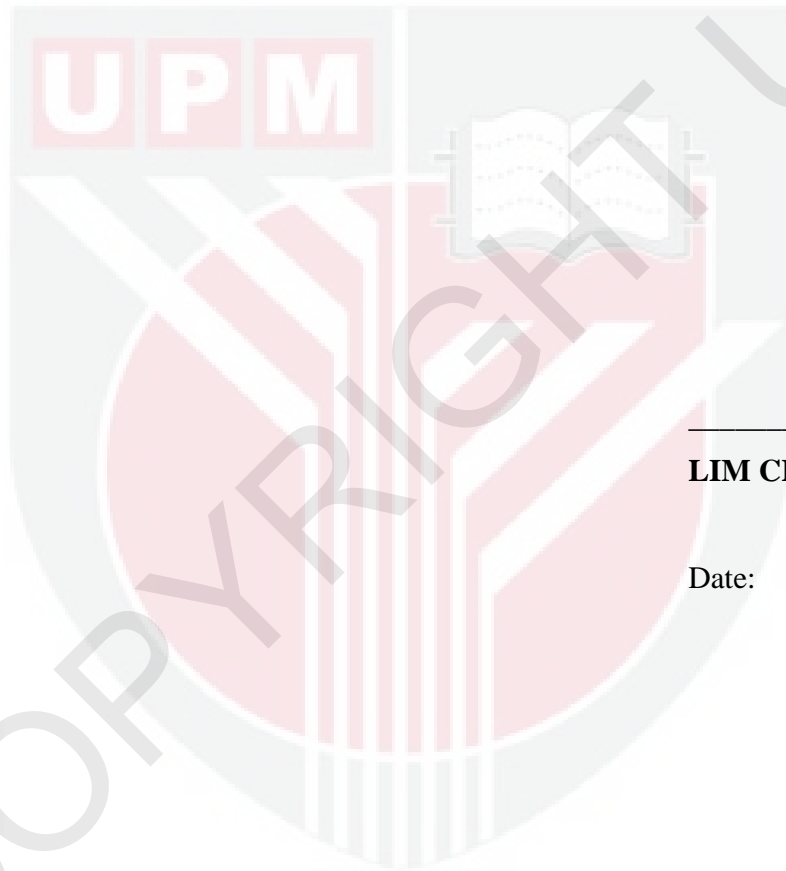
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DECLARATION

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.



LIM CHOOI LING

Date:

TABLE OF CONTENTS

DEDICATION	Page
ABSTRACT	ii
ABSTRAK	iii
ACKNOWLEDGEMENTS	vi
APPROVAL	ix
DECLARATION	x
LIST OF TABLES	xii
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xviii
	xxix

CHAPTER

1	INTRODUCTION	1
2	LITERATURE REVIEW	4
2.1	Liver Fibrosis	4
2.1.1	Epidemiology and pathogenesis of liver fibrosis	5
2.1.2	Diagnostic options in liver fibrosis	9
2.1.3	Hepatic stellate cells (HSCs)	10
2.1.4	Treatment strategies of liver fibrosis	28
2.2	Transforming growth factor- β 1 (TGF- β 1)	34
2.2.1	TGF- β -induced Smad-dependent signaling and crosstalk with MAP kinase pathway	36
2.2.2	Pathological role of TGF- β (carcinogenesis)	40
2.2.3	Role of TGF- β in liver fibrosis	42
2.2.4	Anti- TGF- β 1 therapeutic strategies against liver fibrosis	45
2.2.5	SB 431542 T β RI inhibitor	50
2.3	Natural products and microbial secondary metabolites as therapeutic precursors	53
2.3.1	Actinomycetes	57
2.3.2	<i>Streptomyces</i> as a source of bioactive compounds	59
2.3.3	Novel <i>Streptomyces</i> sp. strain H6552 with therapeutic potential	61

3	GENERAL METHODOLOGY	66
3.1	Cell culture	66
3.2	³ H-Thymidine incorporation assay	67
3.3	DGA-2 luciferase assay	69
3.4	RNA extraction and reverse transcription (RT)	70
3.5	Real-Time PCR	72
3.6	Western Blot (Immunoblotting)	76
3.7	Plasmids	79
3.7.1	Large-scale isolation and purification of plasmid DNA (Maxiprep)	79
3.7.2	Transfection with Smad reporter plasmid	81
3.7.3	Luciferase assay	81
3.8	Immunocytochemistry	82
3.9	Oil Red O intracellular lipid staining	83
3.10	Statistical analysis	84
4	MORPHOLOGICAL AND PROTEIN EXPRESSION OF SB 43152 ON HUMAN HEPATIC STELLATE CELLS	85
4.1	Introduction	85
4.2	Materials and Methods	89
4.2.1	Matrigel coating	90
4.2.2	Growth curve analysis	90
4.2.3	Phase contrast microscopy- morphological analysis	91
4.2.4	Oil Red O staining	91
4.3	Results	92
4.3.1	Growth pattern and cellular proliferation changes induced by TGF- β 1 and/or SB 431542	92
4.3.2	SB 431542-induced inhibition of TGF- β 1 signaling and modulation of LX-2 gene and protein expression	97
4.3.3	Effect of Matrigel on LX-2 quiescence	107
4.4	Discussion	116
4.4.1	Effect of TGF- β 1 on the growth pattern, proliferation, and activation of LX-2 HSC	116

4.4.2	Effect of SB 431542 on TGF- β 1-induced signaling, proliferation, and activation of LX-2 cells	117
4.4.3	Activation of HSC in prolonged culture	124
4.4.4	Effect of Matrigel on LX-2 quiescence	125
4.5	Conclusion	129
5	ANTIFIBROTIC ACTIVITIES OF <i>STREPTOMYCES</i> SP. STRAIN H6552 EXTRACT ON HUMAN HEPATIC STELLATE CELLS	131
5.1	Introduction	131
5.2	Materials and Methods	135
5.2.1	Culture of <i>Streptomyces</i> sp. H6552	136
5.2.2	Fermentation and acetone extraction of secondary metabolites (aerobic culture)	136
5.2.3	Freeze-drying and storage of extract	137
5.2.4	High-performance liquid chromatography (HPLC) and fraction collection	137
5.2.5	Cell line treatment with H6552 extract or fractions	138
5.2.6	MTT cell viability assay	139
5.2.7	MitoTracker Red® fluorescence staining (analysis of oxidative stress)	141
5.2.8	Transfection efficiency analysis (GFP)	141
5.3	Results	143
5.3.1	Culture and HPLC fractionation of <i>Streptomyces</i> sp. (H6552) extract	143
5.3.2	Effects of H6552 crude extract and F3 on cellular response	150
5.3.3	Inhibition of H6552 crude extract and F3 on TGF- β 1-induced Smad3 signaling	162
5.3.4	Effects of H6552 crude extract and F3 on modulation of LX-2 molecular signaling	171
5.4	Discussion	193
5.4.1	Culture, extraction, and fractionation of H6552 crude extract	193

5.4.2	Effect of crude extract and F3 on DGA-2 luciferase activity	195
5.4.3	Effect of crude extract and F3 on cell viability and proliferation	197
5.4.4	Mitochondrial stress attenuation in LX-2 cells by H6552 extract treatment	199
5.4.5	Induction of an adipocytic phenotype in F3-treated LX-2	201
5.4.6	Transfection efficiency of LX-2 and dermal fibroblasts	202
5.4.7	Attenuation of Smad reporter luciferase by H6552 extract and F3	205
5.4.8	Gene and protein regulation of LX-2 cells by crude extract and F3 treatment	207
5.4.9	Effect of H6552 extract and F3 on intracellular α -sma expression	214
5.5	Conclusion	216
6	SUMMARY, GENERAL CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH	218
6.1	Summary and general conclusion	218
6.2	Limitations of the study and recommendations for future research	220
	REFERENCES	223
	APPENDICES	262
	BIODATA OF STUDENT	275