UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM STREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER AGENT

MASRIANA HASSAN

FPSK(M) 2007 18
IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM
STREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER
AGENT

By

MASRIANA HASSAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia
in Fulfillment of the Requirement for the Degree of Master of Science

February 2007
Specially dedicated to,

My husband, Mohd. Solvee, son, Muhammad Sofwan Wazeen, daughter Sofea Adriana and parents, Hassan and Sapura

For their love, understanding, encouragement, patience and moral support.
IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM STREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER AGENT

By

MASRIANA HASSAN

February 2007

Chairman: Professor Seow Heng Fong, PhD

Faculty: Medicine and Health Sciences

Aberrations in the phosphatidylinositol 3-kinase (PI3K)/Akt pathway have been found in a wide spectrum of human cancers. Activation of Akt and inactivation of the downstream substrates such as GSK-3β, BAD and Forkhead family (FKHR) proteins are relevant to promote cell survival, proliferation and growth. Another related pathway linked with PI3K/Akt is the Ras/Raf-1/MEK/ERK, which is known to promote cancer as a result of ras-transformation. The discovery of new drugs targeted at specific molecules of these pathways is a 'hot' field in cancer research. Blocking the constitutively active PI3K/Akt pathway provides a new strategy for cancer therapy. Thus, inhibitors of this signaling pathway would be potential anti-cancer agents. The Streptomyces strain H7372 isolated from mangrove soils in Sabah was found to inhibit the Ras/Raf-1 protein interaction in the yeast two-hybrid screening system. The present study was undertaken to determine the cytotoxic effect of H7372 fractionated extract on a breast cancer cell line, MCF-7 and a non-tumorigenic epithelial cell line, MCF-10A and quantitatively measure kinase inhibition, apoptosis induction and cell cycle disruption. The crude
extracts of H7372 were fractionated into eight fractions using reverse phase HPLC. Fraction 5 was found to be the most cytotoxic in an MTT assay. The crude extract and fraction 5 of H7372 were found to exert growth inhibition of MCF-7 at IC$_{50}$ of 15µg/ml and 1.4 µg/ml, respectively. Western blot analyses showed that activated PI3K and Akt (Thr308) but not Akt (Ser473) by stimulation of IGF-I were inhibited by the crude extract and fraction 5 after 72 hours. Interestingly, phosphorylation of Raf-1 (Ser259) and ERK1 were also inhibited by fraction 5, indicated that there is a cross-talk between PI3K/Akt and MAPK pathways. By using the flow cytometry technique, we found that fraction 5 inhibited the proliferation of MCF-7 cell line by causing them to arrest in the G$_1$ phase of the cell cycle. The induction of growth arrest by fraction 5 was associated with accumulation of cells in G$_1$ and decreasing cells in S and G$_2$/M phases. The results were supported by inhibition of cyclin D1 in MCF-7 cells. The apoptosis study showed that fraction 5 but not crude extract was increased the percentage of cells in early apoptotic stage at all concentrations. Furthermore, treatment of MCF-7 cells with fraction 5 resulted in reduction in phosphorylation of GSK-3β (Ser9), phospho-BAD (Ser112) and phospho-FKHR (Ser256). These results could contribute the apoptosis in MCF-7 cell line. Thus, we discovered that fraction 5 of H7372, a naturally occurring microbe, contains an inhibitor of cell proliferation, cell cycle progression and is able to induce apoptosis via the PI3K/Akt and MAPK pathways.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN AKTIVITI PERENCAT PROTEIN KINASE DARIPADA STREPTOMYCES STRAIN H7372 BAGI KEGUNAAN YANG BERPOTENSI SEBAGAI AGEN ANTI-KANSER

Oleh
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Februari 2007

Pengerusi: Profesor Seow Heng Fong, PhD
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Gangguan dalam lintasan phosphatidylinositol 3-kinase (PI3K)/Akt (Protein Kinase B) telah ditemui secara meluas di dalam kanser manusia. Pengaktifan Akt dan penyahaktifan substrat bawahan seperti protein GSK-3β, BAD and Forkhead family (FKHR) adalah berkaitan bagi pemanjangan hayat, penambahan dan pertumbuhan sel. Lintasan lain yang berkaitan dengan PI3K/Akt adalah Ras/Raf/MEK/ERK di mana ia telah dikenalpasti bagi menyebabkan kanser yang disebabkan oleh transformasi raf.

Penemuan ubat-ubatan baru bagi sasaran molekul yang spesifik untuk lintasan ini adalah bidang yang hangat dalam kajian kanser. Penghalangan lintasan PI3K/Akt yang aktif berterusan memberi starategy baru untuk terapi kanser. Oleh itu, perencat lintasan isyarat ini akan menjadi agen antikanser yang berpotensi. Streptomyces strain H7372 yang diasingkan daripada tanah paya bakau di Sabah telah merencat interaksi protein Ras/Raf-1 di dalam sistem penyaringan ‘yeast two-hybrid’. Kajian ini telah dilakukan untuk menentukan kesan sitotoksik oleh ekstrak H7372 yang telah difraksi terhadap sel kanser payudara, MCF-7 dan sel epithelium bukan tumor, MCF-10A, serta mengukur secara
kuantiti perencatan kinase, induksi apoptosis dan pemuasah kitaran sel. Ekstrak mentah H7372 telah difraksikan kepada lapan fraksi menggunakan fasa berbalik HPLC. Fraksi ke-5 telah dikenalpasti sebagai fraksi yang paling sitotoksik di dalam asei MTT. Ekstrak mentah telah dikenalpasti dapat merencat pertumbuhan pada IC50 15µg/ml manakala, fraksi ke-5 pada 1.4 µg/ml. Analisis dari kaedah 'Western blot' telah menunjukkan bahawa pengaktifan PI3K dan Akt (Thr308) tetapi bukan Akt (Ser473) dengan rangsangan IGF-I telah direncat oleh ekstrak mentah dan fraksi ke-5 selepas 72 jam. Fosforilasi Raf-1 (ser259) dan ERK 1/2 juga telah direncat oleh fraksi ke-5 dan ianya menunjukkan bahawa terdapatnya hubungan antara lintasan PI3K/Akt dan lintasan MAPK. Dengan menggunakan teknik 'flow cytometry', kami mendapati bahawa fraksi ke-5 telah merencat penambahan sel MCF-7 dan dengan ini menyebabkan sel tersebut ditaikan pada fasa G1 di dalam kitaran sel. Induksi bagi penahanan pertumbuhan oleh fraksi ke-5 telah menyebabkan pengumpulan sel di dalam fasa G1 dan mengurangkan jumlah sel di dalam fasa S dan G2/M. Keputusan-keputusan ini telah disokong oleh perencatan cyclin D1 dalam sel MCF-7. Kajian apoptosis telah menunjukkan bahawa fraksi ke-5 tetapi bukan ekstrak mentah telah meningkatkan peratusan sel pada tahap awal apoptosis bagi setiap kepekatan. Tambahan pula, rawatan sel MCF-7 oleh fraksi ke-5 telah menyebabkan penurunan di dalam fosforilasi GSK-3β (Ser9), BAD (Ser112) dan FKHR (Ser256). Keputusan-keputusan ini boleh menyumbang kepada apoptosis bagi sel MCF-7. Oleh itu, kajian ini telah mengenalpasti bahawa fraksi ke-5 daripada mikroorganisma semulajadi mengandungi perencat bagi penambahan sel, progresi kitaran sel dan juga berupaya untuk merangsang apoptosis melalui lintasan PI3K/Akt dan MAPK.
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Prof. Dr. Seow Heng Fong for her invaluable guidance, encouragement and endless support throughout this study. Her careful review and constructive criticism have been crucially important for this thesis.

Special thanks go to my co-supervisors, Prof. Ho Coy Choke, Dr. Maha Abdullah and Dr. Anthony Ho Siong Hock for their advices and assistance throughout the entire progress of this study.

My sincere thanks go to Dr. Khor Tin Oo, Cheah Hween-Yee, Foo Sek Hin, Puah Seok Hwa, Lim Pei Ching, Leong Pooi Pooi, Loh Hui Woon and Yip Wai Kien, thank you for being caring and readily assisting me when I need it most.

I am indebted to my labmates, See Hui Shien, Leslie Than, Jee Jap Meng, Vincent, Siti Aishah and Mr. Anthonysamy for their collaboration and sharing everyday joys and miseries in the making of science.

Last but not least, I would like to express my heartfelt gratitude to my husband, Mohd. Solvee, thank you for his love and patient, encouragement and endless support throughout this study. Especially thanks to my beloved parents for their understanding and support during the entire study in UPM.
I certify that an Examination Committee has met on 27th February 2007 to conduct the final examination of Masriana Binti Hassan on her Master of Science thesis entitled “Identification of Protein Kinase Inhibitory Activities from *Streptomyces* Strain H7372 for Potential Use as Anti-Cancer Agent” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 10 MAY 2007
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

[Signature]

MASRIANA HASSAN

Date: 1st AUGUST 2007
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4.15 Comparison line chart on the normalized level of expression of phospho-GSK-3β (Ser9), as compared to baseline of total GSK-3β; and phospho-BAD (Ser136) and phospho-FKHR (Ser256) as compared to baseline of β-actin. Graphical data represent the MCF-7 cell line was treated and untreated with fraction 5 at various concentrations for 72 hours.
4.16 Effects of H7372 fraction 5 on phospho-ERK1/2 and phospho-Raf (Ser259). MCF-7 cell line was treated and untreated with various concentrations of fraction 5 for 72 hours and stimulated with IGF-I.

4.17 Comparison line chart on the normalized level of expression of phospho-ERK1/2 and phospho-Raf (Ser259) as compared to baseline total ERK and Raf respectively. Graphical data represent the MCF-7 cell line was treated and untreated with various concentrations of fraction 5 for 72 hours.

4.18 Effects of crude extract, H7372 on the cell cycle profile. Histogram shows the phases in the cell cycle: G1 (M1); S (M2); G2/M (M3) and Sub-G1 (M4). The MCF-7 cells were cultured in medium alone as a control or cultured in the presence of H7372 for various concentrations for (A) 24 hours, (B) 48 hours and (C) 72 hours. I; Control, II; 7.5 µg/mL H7372, III; 15 µg/mL H7372, IV; 30 µg/mL H7372.

4.19 Percentage of MCF-7 cells in G1 phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in G1 phase which were treated with the H7372 extract slightly increased at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05.

4.20 Percentage of MCF-7 cells in S phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in S phase which were treated with the H7372 extract decreased at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05.

4.21 Percentage of MCF-7 cells in G2/M phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in G2/M phase which were treated with the H7372 extract slightly decreased at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar.

4.22 Percentage of MCF-7 cells in Sub-G1 phase treated with the crude extracts of H7372 based on DNA content in two independent experiments. The percentage of cells in Sub-G1 phase which were treated with the H7372 extract have no significant different at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar.
Effects of fraction 5, H7372 on the cell cycle profile. Histogram shows the phases in the cell cycle: G1 (M1); S (M2); G2/M (M3) and Sub-G1 (M4). The MCF-7 cells were cultured in medium alone as a control or cultured in the presence of fraction 5 for various concentrations for (A) 24 hours, (B) 48 hours and (C) 72 hours. I; Control, II; 0.7 µg/mL Fraction 5, III; 1.4 µg/mL Fraction 5, IV; 2.8 µg/mL Fraction 5.

Percentage of MCF-7 cells in G1 phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in G1 phase which were treated with the fraction 5 shows significant increase at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05.

Percentage of MCF-7 cells in S phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in S phase which were treated with the fraction 5 shows significant decrease at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05.

Percentage of MCF-7 cells in G2/M phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in G2/M phase which were treated with the fraction 5 shows significant decrease at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05.

Percentage of MCF-7 cells in Sub-G1 phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in Sub-G1 phase which were treated with the fraction 5 shows significant increase after 72h incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05.

Effects of fraction 5 on the cell cycle profile. Histogram shows the phases in the cell cycle: G1 (M1); S (M2); G2/M (M3) and Sub-G1 (M4). The MCF-10A cells were cultured in medium alone as a control or cultured in the presence of fraction 5 in various concentrations for (A) 24 hours, (B) 48 hours and (C) 72 hours. I; Control, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

Percentage of MCF-10A cells in G1 phase treated with fraction 5
based on DNA content in two independent experiments. The percentage of cells in G\(_1\) phase which were treated with the fraction 5 shows no significant increase at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar.

4.30 Percentage of MCF-10A cells in S phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in S phase which were treated with the fraction 5 shows significant decrease after 24h incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05.

4.31 Percentage of MCF-10A cells in G\(_2/M\) phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in G\(_2/M\) phase which were treated with the fraction 5 shows significant decrease at 2.8μg/mL after 48h incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar. *, indicate significantly lower than control, p < 0.05.

4.32 Percentage of MCF-10A cells in Sub-G\(_1\) phase treated with fraction 5 based on DNA content in two independent experiments. The percentage of cells in Sub-G\(_1\) phase which were treated with the fraction 5 have no significant different at all incubation period compared to the untreated cells (control). The results shown were mean ± 1 SD bar.

4.33 Effects of fraction 5 on cyclin D1. MCF-7 cell line was with and without fraction 5 at various concentrations for 72 hours and stimulated with IGF-I.

4.34 Comparison line chart on the normalized level of expression of cyclin D1 as compared to baseline β-actin. The expression level of cyclin D1 was decreased by fraction 5 in a dose-dependent manner. Graphical data represent the MCF-7 cell line was treated and untreated with fraction 5 in various concentrations for 72 hours.

4.35 MCF-7 treated with H7372 for 24 hours. The cells were stained with Annexin V and PI. I; 0 μg/mL H7372, II; 7.5 μg/mL H7372, III; 15 μg/mL H7372, IV; 30 μg/mL H7372.

4.36 MCF-7 treated with H7372 for 48 hours. The cells were stained with Annexin V and PI. I; 0 μg/mL H7372, II; 7.5 μg/mL H7372, III; 15 μg/mL H7372, IV; 30 μg/mL H7372.

4.37 MCF-7 treated with H7372 for 72 hours. The cells were stained with
Annexin V and PI. I; 0 µg/mL H7372, II; 7.5 µg/mL H7372, III; 15 µg/mL H7372, IV; 30 µg/mL H7372.

4.38 Percentage of (A) early apoptotic cells, (B) late apoptotic cells, and (C) necrotic cells in two independent experiments. MCF-7 cells were treated with crude extracts of H7372 at indicated time and concentrations. The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05.

4.39 MCF-7 treated with fraction 5 for 24 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

4.40 MCF-7 treated with fraction 5 for 48 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

4.41 MCF-7 treated with fraction 5 for 72 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

4.42 Percentage of (A) early apoptotic cells, (B) late apoptotic cells, and (C) necrotic cells in two independent experiments. MCF-7 cells were treated with fraction 5 at indicated time and concentrations. The results shown were mean ± 1 SD bar. *, indicate significantly greater than control, p < 0.05.

4.43 MCF-10A treated with fraction 5 for 24 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

4.44 MCF-10A treated with fraction 5 for 48 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

4.45 MCF-10A treated with fraction 5 for 72 hours. The cells were stained with Annexin V and PI. I; 0 µg/mL fraction 5, II; 0.7 µg/mL fraction 5, III; 1.4 µg/mL fraction 5, IV; 2.8 µg/mL fraction 5.

4.46 Percentage of (A) early apoptotic cells, (B) late apoptotic cells, and (C) necrotic cells. MCF-10A cells were treated with fraction 5 at indicated time and concentrations.

5.1 Regulation of the G1/S phase transition (Adapted from Roy and Thompson, 2006).
**LIST OF ABBREVIATIONS**

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>~</td>
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<td>°C</td>
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<td>American Type Cell Collection</td>
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<td>BSA</td>
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