UNIVERSITI PUTRA MALAYSIA

CYTOTOXICITY MECHANISM OF A FUNGAL INHIBITOR FROM A SOIL-DERIVED *STREPTOMYCES* SP.

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By

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

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

CYTOTOXICITY MECHANISM OF A FUNGAL INHIBITOR FROM A SOIL-DERIVED *STREPTOMYCES* SP.

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Chairman : Professor Seow Heng Fong, PhD

Faculty : Medicine and Health Sciences

Effective fungal growth inhibitors are important to drive the development of antifungal compound. In the search for fungal inhibitors, actinomycete H7372 was isolated from a mangrove soil sample from Sabah. Subsequently, in a yeast cell-based screening system, the crude acetone extract prepared from the fermentative culture of H7372 was found to inhibit the growth of the yeasts. The purposes of this study were to establish the phylogenetic position of H7372 and to isolate, characterize and examine the toxicity mechanism of the active fungal growth inhibitor produced by H7372. The partial sequence of 16S rRNA gene was amplified from H7372 for phylogenetic analysis. An active compound was isolated from crude acetone extract of mannitol-soybean fermentation culture and its structure was elucidated. Antifungal properties of the isolated active compound against *Candida* spp and *Aspergillus* spp were characterised by minimum inhibitory concentration and time-kill kinetic studies. The consequences of *C. glabrata* treatment with the active compound were examined by electron microscopy and cDNA microarray. Phylogenetic analysis placed H7372 to its closest relative, *S. kasugaensis* M338-M1.

The isolated active compound, designated as J5, was determined to be the natural (11S, 13S, 9S, 8R)-cycloheximide. All *Candida* species tested (except *C. albicans*) and only *A. niger* were sensitive to J5, with MIC at 24H ranging from 0.313 to 40 µg/ml. The degree of susceptibility shown by some species of *Candida*, from the most to the least susceptible, were *C. krusei*, *C. glabrata*, *C. rugosa* and *C. parapsilosis*. J5 is a fungistatic compound which showed total fungicidal effect at 12 times of its MIC when applied to *C. glabrata*. Treatment with J5 demonstrated profound intracellular and cell surface modifications, such as by marked cell wall thickening, confused cytoplasm, mitochondria loss, and irregular plasma membrane invaginations with detachment of the protoplast from the cell wall. cDNA microarray revealed a total of 60 genes affected by J5 treatment, corresponding to genes involved in protein synthesis, plasma membrane and H^+ pumps, mitochondria maintenance and nutrient metabolism. In conclusion, H7372 is a *Streptomyces* sp. which is closely related to *S. kasugaensis* M338-M1. The active compound, J5, is a *cis*-cycloheximide. Comprehensive susceptibility profiles of *Candida* and *Aspergillus* species toward J5 were established for the first time and generated new MIC readings for *C. krusei* (0.313 µg/ml), *C. rugosa* (0.625 µg/ml), *C. glabrata* (2.5 µg/ml), *C. parapsilosis* (2.5 µg/ml), *C. tropicalis* (5 µg/ml) and *A. niger* (40 µg/ml). Ultrastructures of J5-treated *C. glabrata* revealed new evidence on the toxicity mechanisms of cycloheximide on plasma membranes and mitochondria. The gene expression profiles for J5, the cycloheximide treatment, were revealed for the first time in yeast.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

MEKANISMA SITOTOSIKSITI PADA SATU PERENCAT KULAT DARIPADA *STREPTOMYCES* SPESIS TANAH

Oleh

JEE JAP MENG

JULAI 2010

Pengerusi : Profesor Seow Heng Fong, PhD

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Bahan perencat pertumbuhan kulat yang berkesan adalah penting untuk menerajui perkembangan kompaun anti-kulat. Dalam usaha pencarian bahan perencat pertumbuhan kulat, aktinomysit H7372 telah dipencil daripada tanah di negeri Sabah. Proses penyaringan yang menggunakan yis dua hybrid mendapati ekstrak mentah fermentasi H7372 boleh merencat pertumbuhan yis. Justeru itu, kajian ini bertujuan untuk megetahui filogeni H7372 dan untuk memencil, memeriksa mekanisma tosiksiti bahan aktif yang dihasilkan daripada H7372. Gen 16S RNA telah diamplifikasi daripada H7372 sebagai bahan genetik untuk kajian filogeni. Satu bahan aktif telah dipencil daripada ekstrak mentah aseton kultur fermentasi mannitol-kacang soya dan struktur kimia bahan aktif ini juga ditentukan. Kesan perencatan antikulat bahan aktif kepada spesis *Candida* dan *Aspergillus* ditentukan sebagai kepekatan perencatan mimima (MIC) dan kinetik perencatan semasa. Kesan rawatan bahan aktif pada *C. glabrata* telah diperiksa melalui mikroskop elektron. Analisis filogenetik mnedapati spesis saudara terdekat kepada H7372 adalah *S. kasugaensis* M338-M1. Analisis struktur kimia mendapati bahan aktif yang telah dipencil, yang dinamakan sebagai J5 adalah *cis*-cycloheximide. Semua spesis *Candida* (kecuali *C. albicans*) dan hanya *Aspergillus niger* adalah sensitif kepada J5, dengan MIC 24 jam

berjulat daripada 0.313 ke 40 µg/ml. Darjah sensitiviti yang disusun daripada spesis paling sensitif adalah seperti *C. krusei*, *C. glabrata*, *C. rugosa* dan *C. parapsilosis*. J5 bersifat fungistatik, hanya menjangkal pembunuhan sepenuhnya pada kepekatan 12 kali MICnya. Rawatan J5 pada *C. glabrata* menunjukkan kesan-kesan modifikasi yang drastik pada luar permukaan sel and intrasellular seperti penebalan dinding sel, kerosakan sitoplasma, kehilangan mitokondria dan juga invaginasi pada plasma membran yang abnormal dengan susutan protoplas daripada dinding cell. Mikroarrai cDNA memaparkan 60 gen terkait dengan rawatan J5, gen-gen ini diklasifikasi kepada sintesis protein, plasma membran dan pam proton, penyelengaraan mitokondria dan metabolisme nutrien. Kesimpulannya, H7372 merupakan spesis Streptomyces yang berkait rapat dengan *S. kasugaensis* M338-M1. Kompaun aktif, J5 adalah cis-cycloheximide. Profail kepekaan komprehensif spesis-spesis *Candida* dan *Aspergillus* kepada cycloheximide semulaji telah ditentukan buat kali pertama dan menghasilkan nilai MIC baharu kepada *C. krusei* (0.313 µg/ml), *C. rugosa* (0.625 µg/ml), *C. glabrata* (2.5 µg/ml), *C. parapsilosis* (2.5 µg/ml), *C. tropicalis* (5 µg/ml) dan *A. niger* (40 µg/ml). Ultrastruktur *C. glabrata* selepas rawatan J5 menunjukkan bukti-bukti baharu mekanisma tosiksiti cycloheximide pada plasma membran dan mitokondria. Profil ekpresi gen J5, iaitu rawatan cycloheximide telah diwujudkan buat kali yang pertama.

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I certify that Examination Committee met on _____ to conduct the final examination of Jee Jap Meng on his Doctor of Philosophy thesis entitled "Cytotoxicity Mechanism of a Fungal Inhibitor from a Soil-Derived *Streptomyces* sp." in accordance with the 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:

_____
(Chairperson)

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(Internal Examiner I)

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

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Date: 25 November 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 other institutions.

_____

JEE JAP MENG

Date: 30 July 2010

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