UNIVERSITI PUTRA MALAYSIA

EFFECT OF COMBINED FRACTION S1 AND GM-H-1 (CITRININ) FROM PENICILLIUM SP. H9318 ON INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS IN HT-29 CELL LINE

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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

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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Microfungi have been much sought after for its secondary metabolites to yield bioactive compounds that are widely investigated for its versatile usage in pharmaceutical areas. Recently, the production of small molecular inhibitors, particularly the inhibitors of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), are under intensive research in pursuit of its efficacy in the cancer therapeutic modalities. In our study, secondary metabolites were isolated from microfungi H9318, a *Penicillium sp*. H9318 was determined as a new strain of *Penicillium citrinum* as shown by the phylogenic study. Two fractions, S1 and GM-H-1, were isolated from H9318 strain and GM-H-1 was later structurally confirmed as Citrinin (CTN). Our

study aims to elucidate cytotoxic mechanism of S1 and CTN on colon cancer cells, HT-29. It is known that CTN is a mycotoxin associated with hepatotoxicity and nephrotoxicity. Interestingly, CTN has also been reported to induce cell cycle arrest and apoptosis in various types of cells, including cancer cells. In an effort to attenuate the toxicity of CTN, a combination of S1 and CTN (S1 + CTN) was commenced to allow synergistic effect on the approach of anticancer regimen. Much evidence on the morphological, colorimetric and FACS (Fluorescent-activated cell sorting) assessment have reflected the pronounced inhibitory effect of S1 and CTN in combination on cell proliferation and mitotic progression. Together with the Western blot analysis, the level of phosphorylated-Extracellular-signal-regulated kinase (p-ERK1/2) was found elevated and phosphorylated-Retinoblastoma protein (p-RB) was decreased, indicating a possible activation of both proteins that is partly responsible for the mechanism in triggering G_2/M phase cell cycle arrest as a consequence of S1 + CTN exposure. Luminogenic assay has shown suppression of Caspase 3/7 activity in an event of apoptosis occurred on HT-29 cells induced by S1 + CTN treatment. In line with this, FACS-analysed activation of pan-caspases was correlated with the induction of p-ERK1/2 and it is speculated that activity of ERK1/2 is maintained by certain caspases which in fact remains to be elucidated. Significantly, our data has provided an alternative agent which could be recommended in the study of cellular

signal transduction pathway, that is, S1 as being recognised as a candidate PP1/PP2A inhibitor throughout a series of composite biochemical assays. However, it should be confirmed by more in depth studies and structural analysis. Presented here is the first report on the toxicity mechanism of CTN on HT-29 cells and presumably a cell-based model entity of the mode of action of S1 + CTN was described. In conclusion, S1 + CTN exerted anticancer activity on HT-29 cells by modulating the signaling pathways of ERK1/2 and RB proteins regulating cell cycle progression and apoptosis independent of Caspase 3/7.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEBERKESANAN CAMPURAN S1 DAN GM-H-1 (CITRININ) DALAM USAHA MENGHENTIKAN PROSES PEMBELAHAN SEL DAN MENYEBABKAN APOPTOSIS PADA SEL HT-29

Oleh

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Kulat merupakan sejenis mikro-organisma eukariot yang boleh digunakan sebagai sumber semula jadi dalam proses penapaian untuk menghasilkan metabolik sekunder yang boleh diproses and diekstrak untuk kepelbagaigunaan dalam bidang farmaseutikal. Kebalakangan ini, penyelidikan dan penghasilan inhibitor bagi molecular kecil, umpamanya inhibitor bagi protein phosphatase 1 (PP1) dan protein phosphatase 2A (PP2A) berkembang pesat dan memberi manfaat kepada industri dan kajian saintifik dalam bidang kanser, seperti strategi yang mengutamakan terapi molekular. Seperti yang dipaparkan atas tesis ini, metabolik sekunder diperolehi daripada penapaian H9318, sejenis kulat, *Penicillium sp*. H9318 telah dikenalpasti sebagai strain baru bagi *Penicillium citrinum* dalam

experiment filogeni. S1 dan GM-H-1 merupakan komponen-komponen bioaktif diperolehi daripada H9318 strain. Experimen kami bertujuan untuk mengkaji mekanisme bagi S1 dan GM-H-1 dalam proses anti-kanser. GM-H-1 telah dikenalpasti sebagai Citrinin (CTN) melalui kajian dari segi struktur dan ia mempunyai sifat toksik terhadap hati dan buah pinggang. Walaubagaimanapun, CTN turut berkesan untuk mempengaruhi sifat-sifat sel dalam proses pembelahan sel (mitosis) dan seterusnya menyebabkan pelbagai jenis sel mengalami apoptosis, termasuk sel kanser. Dalam usaha untuk merendahkan ketoksikan CTN, campuran S1 dan CTN dalam satu regimen teleh dijalani dalam praktis bagi menunjukkan keberkesanan regimen itu pada sel kanser kolon, HT-29. Data hasil kerja kami telah mengemukakan bahawa S1 dan CTN boleh berkerjasama untuk peningkatan efikasi regimen, keadaan ini digelar sinergi interaksi. Kajian morfologi, pembentukan warna dan FACS (Fluorescent-activated cell sorting) telah mencatatkan bioefikasi bagi S1 + CTN mengenai kemerosotan sel dari segi mitosis dan apoptosis. Sehubungan dengan itu, teknologi Western blot telah memaparkan data tentang kenaikan phosphorylated-Extracellular-signal-regulated kinase (p-ERK1/2) dan penurunan phosphorylated-Retinoblastoma protein (p-RB) dalam sel selepas inkubasi dengan S1 + CTN. Pada hal yang demikian, pengaktifan protein ERK1/2 dan RB mungkin terbabit dalam penyelisihan rangkaian isyarat dalam system sel. Sejajar dengannya, kajian

luminogenik merekodkan penurunan activiti pada Caspase 3/7 yang selaras dengan kelakuan apoptosis. Analisis bagi FACS pada keseluruhan caspase dalam sel juga memberi spekulasi bahawa aktiviti bagi ERK1/2 dikawal oleh sesetengah caspase. Secara signifikannya, S1 berpotensi untuk dijadikan sebagai satu agen bagi kajian rangkaian isyarat dalam sel sehubungan dengan sifat anti-PP1/PP2A. Pertama sekali, kertas kerja ini menunjukkan satu model entiti yang berkaitan dengan mekanisme yang dipaparkan oleh S1 + CTN pada sel HT-29. Kesimpulannya, hasil penyelidikan kami sekurang-kurangnya menunjukkan bahawa sel kanser kolon bertindak balas secara pemerosotan dari segi mitosis dan apoptosis bersamaan dengan peningkatan activiti bagi protein ERK1/2 dan RB yang bebas daripada Caspase 3/7.

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I certify that an Examination Committee has met on 11th March 2010 to conduct the final examination of Ooi Suek Chin on her thesis entitled "**Effect of combined Fraction S1 and GM-H-1 (Citrinin) from *Penicillium sp.* H9318 on induction of cell cycle arrest and apoptosis in HT-29 cell line**" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis 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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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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OOI SUEK CHIN

Date:

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5-FU	5-Fluorouracil
AD	Alzheimer's disease
AKAP149	A-kinase anchoring protein
AO	Acridine orange
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
AV	Annexin V
BAD	Bcl-2 antagonist of cell death
BCL-2	B-cell lymphoma/leukemia 2
BCL-xL	B-cell lymphoma-extra large
BID	BH3 interacting domain death agonist
Bp	base pair
BSA	Bovine serum albumin
CA	Calyculin A
CAK1	CDK-activating kinase
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
CANT	Cantharidin
CDC2	Cell division cycle 2
CDC25	Cell division cycle 25
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CPM	Count per minute
CTN	Citrinin
CTS	Cytostatin
DD	Death domain
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E
ERK1/2	Extracellular-signal-regulated kinase
EtBr	Ethidium bromide
FACS	Fluorescent-activated cell sorting
FADD	Fas-associated via death domain

FasL	Fas ligand
FBS	Fetal bovine serum
FOXO1	Forkhead transcription factor FKHR
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
H_2O_2	Hydrogen peroxide
I-1	Inhibitor-1
IL-6	Interleukin-6
ITS-LSU	Internal Transcribed Spacer-Large Subunit Ribosomal RNA gene
kb	kilobase pair
KCa	Ca^{2+}-activated K^+ channel
kD	kilo Dalton
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MC	Microcystin
MDM-2	Mouse double minute-2
$MgCl_2$	Magnesium chloride
min	Minute
MMP	Matrix metalloproteinase
mRNA	messenger Ribonucleic acid
MS-NMR	Mass Spectrometer-Nuclear Magnetic Resonance
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NF-L	Neurofilament-L
NHE	Na/H exchanger
NK cells	Natural killer cells
NO	Nitric oxide
O_{2^-}	Superoxide
OA	Okadaic Acid
OD	Optical density
OS	Oxidative stress
p34CDC2	Phosphorylated-cell division cycle 2 with molecular weight of 34 kD
p42	ERK2
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PI	Propidium iodide
PI3K	Phosphatidylinositol-3 kinase

PKA	cAMP-dependent protein kinase
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP1c	Catalytic subunit of PP1
PP2A	Protein phosphatase 2A
PP2Ac	Catalytic subunit of PP2A
PP1/PP2A	PP1 and PP2A
PP2B	Protein phosphatase 2B
PP2C	Protein phosphatase 2C
PP4	Protein phosphatase 4
PP5	Protein phosphatase 5
PP6	Protein phosphates 6
PP7	Protein phosphatase 7
pTyr	Phosphorylated tyrosine
PVDF	Polyvinylidene difluoride
RB	Retinoblastoma protein
RLU	Unit relative to luciferase activity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSK	Ribosomal S6 Kinase
SARS	Severe Acute Respiratory Syndrome
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sec	Second
ser	Serine
SLE	Systemic lupus erythematosus
SOS	Son of sevenless
SRC-3	Steroid receptor coactivator
STAT	Signal transducers and activators of transcription
STS	Staurosporine
TBS	Tris-buffered saline
thr	Threonine
TM	Tautomycin
TNF	Tumour necrosis factor
TRADD	TNFR1 associated death domain protein
tRNA	transfer Ribonucleic acid
UMS	Universiti Malaysia Sabah
YPD	Yeast Peptone Dextrose
ZVAD-FMK	*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

CHAPTER 1

INTRODUCTION

Mortality index registered under cancer as the cause of death has grasped worldwide attention and the mortality rate seems to be in the rise disregard of region or ethnicity. Exploration in the development of cancer therapeutic drugs is inevitably at this stage and has always been the focus on the effusive discovery of natural resources for anticancer regimens.

Microbial products are much sought after for microbes could be sampled everywhere, from the sites under water, on land or at extreme conditions, such as Antarctic or near volcano. In addition, cultures and maintenance of microbes are easy and could be done in a laboratory without much labour intensive and investment spent, therefore, attention has been placed on the fermentation broth for secondary metabolite production.

Successful identification of the bioactive compounds for the development of antibacterial, antifungal and anticancer agents has been an encouragement for the ongoing research on the area of drug design and synthesis. With the advancement of the knowledge on small molecule inhibitors, for example the

discovery of Okadaic Acid (OA), has diverted vast attention to the implication of protein phosphatase inhibition on cancer cells. Over time, a number of protein phosphatase inhibitors have been discovered and investigated for their anticancer properties and some even are currently under clinical trial as a potential chemotherapeutic agent (Kawada et al., 1999, 2003, 2004; Janssens and Goris, 2001 and McCluskey et al., 2001, 2002a, 2002b, 2003).

Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) belong to the PPP family of serine/threonine (ser/thr) protein phosphatase and constitute 90% of all the eukaryotic activity of ser/thr protein phosphatases (Oliver and Shenolikar, 1998). Much work have been done on the research of bioactivity of PP1 and PP2A (PP1/PP2A) and their structural organisation, interaction between catalytic and regulatory subunits, ionic regulation of substrates specificity, physiological importance of inhibitors and their regulatory roles in signaling transduction could be clarified in many review papers (Hunter, 1995; Cohen, 1997; Schonthal, 1998; Aggen et al., 2000; Bollen, 2001; Cohen, 2002 and Ceulemans and Bollen, 2004).

Notable cell growth and proliferation signaling pathways, such as, PI3K/AKT, MAPK, mTOR and WNT pathways are regulated by PP1 and

PP2A. Besides that, inhibition of PP1 and PP2A could deregulate cell cycle progression and result in cell cycle arrest which could end up in apoptosis. A lot of cell cycle and apoptotic regulatory proteins have been controlled by the phosphatases, including BCL-2 family proteins, MDM-2, p53, Retinoblastoma protein and more. Much to our concern, mRNA and activity of PP1 and PP2A have been found elevated in rat hepatoma and during liver regeneration (Sasaki et al., 1990 and Saadat et al., 1995). Besides that, induction of PP2A was observed in the oncogenic transfectants and the transformed cells were shown with flat reversion after exposure to OA (Sakai et al., 1989 and Rajesh et al., 1999). According to the report of Li et al., PP1 stabilised SRC-3 protein, which is an oncogenic protein, by blocking the proteosome-dependent degradation activity (Li et al., 2008). In view of that, potential therapeutic usage of PP1 and PP2A inhibitors are explosive and not only constrained on targeting the growth and proliferative capacity of a cancer but also implicated in the regulation of anti-metastasis and anti-angiogenesis. To extend our understanding, diversified roles of PP1 and PP2A in the strategy of targeting therapeutic on various cancer cells have been reviewed (Schonthal, 2001; Klumpp and Krieglstein, 2002; McCluskey et al., 2002; Garcia et al., 2003; Eichhorn et al., 2009 and Virshup and Shenolikar, 2009).

Sabah is naturally enriched in flora and fauna and has been an attraction to scientists in searching for the diversity of microbes in the purpose of evolutionary study of bioactive compounds discovery. Microfungus, H9318, has considerably been identified as a new strain of *Penicillium citrinum* following a phylogenetic study, was isolated from the soil under leaflets at Maliau Basin, Sabah, Malaysia. Further preliminary screening tests have determined the extracts of H9318 contain potential inhibitory effect on PP1 and PP2A (Ong et al., 2007). Following purification and spectra studies, the extracts were fractionated and were identified as S1 and Citrinin (CTN). It was exciting to find out that the fractions are cytotoxic to various cancer cell lines and colon cancer cells, HT-29, is much susceptible to the treatments. The identity of S1 is unknown but CTN is a known compound, which is a mycotoxin and highly toxic to liver and kidney of many organisms (Lura et al., 2004; Heussner et al., 2006 and Kumar et al., 2007). Since cancer cells are less sensitive to S1, we would like to investigate whether the combination of S1 and CTN could exert synergistic anti-cancer effect besides fulfilling the requirement of lowering the toxicity of CTN on cells. Based on a multiplexed biochemical assays, S1 and CTN could elicit combinatorial synergistic activity on colon cancer cell proliferation, cell cycle progression and apoptosis. Given the idea that S1 emerges as a potential PP1 and PP2A inhibitor supplemented with our data in which concurrent activation of

ERK1/2 and RB protein are found in mitotic block regulation, have thus expanded our view on the anticipated regulatory roles of S1 + CTN on the modulation of mitotic progression. Furthermore, through a series of studies on the activity of pan-caspase and Caspase 3/7, we could possibly postulate a mechanism of action potentiated by the interactive S1 + CTN on the events of cell cycle arrest and apoptosis. The aim of our study is to investigate the anti-cancer property of S1 and CTN on colon cancer cells. Experiments were carried out based on several hypotheses:

1. S1 and CTN are cytotoxic to cancer cell lines.
2. Combination of S1 and CTN confers synergistic effect.
3. Combinatorial synergism of S1 and CTN enhances toxicity on cell proliferation of colon cancer cells.
4. Signaling pathways of cell cycle progression and apoptosis are disrupted by S1 + CTN.

Objectives of our study are:

1. To determine the inhibitory effect of the extracts of H9318 strain on PP1

2. To investigate whether combination of S1 and CTN would elicit synergism on HT-29 cells

3. To uncover the synergistic effect of S1 and CTN on cell proliferation

4. To check the synergistic effect of S1 and CTN on the signaling pathways of cell cycle progression on HT-29 cells

5. To investigate the synergistic effect of S1 and CTN on the signaling pathways of apoptosis on HT-29 cells

6. To determine the mode of action of S1 + CTN in the process of triggering cell cycle arrest and apoptosis on HT-29 cells

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