UNIVERSITI PUTRA MALAYSIA

CYTOTOXICITY OF GONIOTHALAMIN ON THE HUMAN HEPATOCELLULAR CARCINOMA HEPG2 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 Masters of Science

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DEDICATION

I wish to dedicate this thesis to my mother and father for their love and giving me the genes for research. They have always believed in me and have always encouraged me not only during this master period but throughout life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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October 2009

Chairman: **Noorjahan Banu Mohamedd Alitheen, Ph.D**

Faculty: **Biotechnology and Biomolecular Sciences**

Goniothalamin is a biologically active styrylpyrone derivative isolated from various *Goniothalamus* sp., belonging to the Annonacae family. This plant extract has been reported to be cytotoxic towards several tumor cell lines such as pancreas carcinoma (PANC-1), gastric carcinoma (HGC-27) and breast carcinoma (MCF-7). The purpose of this study was to examine and characterize the *in vitro* cytotoxicity effect of goniothalamin on the human hepatocellular carcinoma HepG2 cells and normal liver Chang cells and also to study the morphological and biochemical changes of goniothalamin-treated HepG2 and Chang cells. Goniothalamin (2.3 -150 µM; 24, 48 and 72 hours) treatment to HepG2 and Chang cells resulted in a dose and time dependent inhibition of cell growth as assessed by MTT and LDH assays. The data suggest that goniothalamin selectively inhibits HepG2 cells (IC_{50} of MTT= 4.6(±0.23) µM; IC_{50} of LDH= 5.20(±0.01) µM for 72 hours) with less inhibition of growth in Chang cells (IC_{50} of MTT= 35.0(±0.09) µM; IC_{50} of LDH= 32.5(± 0.04) µM for 72 hours. The cytotoxic activity of goniothalamin on HepG2 cells was confirmed by Trypan blue dye exclusion

assay. Goniothalamin reduced the number of viable cells (non-stained) associated with an increase on the number of non-viable cells (stained) and the Viability Indexes were 52 ± 1.73% for HepG2 cells and 62 ± 4.36% for Chang cells at IC_{50} after 72 hours. Cells were exposed to goniothalamin at lowest concentration (2.3 μM), IC_{50} (of MTT results), and highest concentration (150 μM) for 24, 48, or 72 hours and then examined for effects on cell cycle (using the flow cytometry) or proliferation (using the BrdU ELISA assay). The cytotoxic activity of goniothalamin was related to the inhibition of DNA synthesis, as revealed by the reduction of BrdU incorporation. At 72 hours with the lowest goniothalamin concentration of 2.3 μM, the normal liver Chang cells retained 97.6% of control proliferation while the liver cancer HepG2 cells were reduced to 19.8% of control proliferation. Goniothalamin caused the accumulation of hypodiploid apoptotic cells in cell cycle analysis by flow cytometry. Goniothalamin arrested HepG2 and Chang cells in the G2/M phase with different degrees. Light microscopy examination of HepG2 and Chang cells exposed to different concentrations of goniothalamin up to 72 h demonstrated changes in cellular morphology; i.e. cell rounding followed by a loss of adherence with subsequent cell shrinkage and blebbing. In addition, the apoptotic cells were more abundant in goniothalamin-treated HepG2 cells (84 ± 4.58%) for 72 hours than in untreated cell (4 ± 2.65%) upon measurement by TUNEL staining. In view of the toxicity of goniothalamin, the kind of cell death, namely apoptosis or necrosis, was assessed. Therefore, staining with fluorescence labeled annexin V in combination with propidium iodide was performed on HepG2 and Chang cells exposed to goniothalamin. The laser scanning cytometry of propidium iodide and annexin V-stained cells indicated that the growth inhibiting effect of goniothalamin was consistent with a strong induction of apoptosis at late stage. This is because the cellular

membrane integrity was lost, so the cells exhibited annexin V- and propidium iodide-double positive up to 85.87 ± 0.78 and 57.69 ± 1.12 in HepG2 and Chang cells after 24 hours, respectively. In order to confirm apoptotic mechanism in the goniothalamin-treated cells, caspase 3 activity upon the same treatment conditions was carried out. The results indicate that caspase 3 activity was significantly elevated early in IC_{50} treated Chang cells (574% of control) after 24 hours and late in IC_{50} treated cells after 72 hours in HepG2 cells (879% of control). Our findings suggest a potential mechanism for the strong growth inhibitory effect of goniothalamin on this HepG2 liver cancer cells. However, less sensitivity to normal liver Chang cell line was observed by this compound. An important feature of the cytotoxicity by goniothalamin is that it is mediated through apoptosis.

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

SITOTOKSISITI GONIOTHALAMIN TERHADAP SEL ASAS KARSINOMA HEPAR HEPG2 PADA MANUSIA

Oleh

MOTHANNA SADIQ OBAID AL-QUBAISI

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Goniothalamin adalah molekul aktif terbitan styrylpyrone secara biologi yang telah diasingpisahkan daripada spesies Goniothalamus dari Famili Annonacea. Ekstrak tumbuhan ini dilaporkan memberi kesan sitotoksik terhadap beberapa sel tumor asas seperti sel karsinoma pankreas (PANC-1), sel karsinoma gastrik (HGC-27) dan sel karsinoma payudara (MCF-7). Tujuan kajian ini adalah untuk memeriksa dan mencirikan kesan sitotoksiti goniothalamin pada sel karsinoma hepar manusia (HepG2) dan sel Chang secara in vitro dan juga mengkaji morfologi dan perubahan biokimia pada sel HepG2 dan sel Chang yang dirawat dengan goniothalamin. Rawatan goniothalamin (2.3-150 μM; 24, 48 dan 72 jam) pada sel HepG2 dan sel Chang dengan menggunakan pengujian MTT dan LDH, menghasilkan keputusan perencatan pertumbuhan sel yang berkadaran dengan dos dan masa. Data mencadangkan goniothalamin merencatkan sel HepG2 (IC_{50} MTT=4.6 (±0.23) μM; IC_{50} LDH=5.20(μM) untuk 72 jam) dengan sedikit perencatan pertumbuhan pada sel Chang (IC_{50} MTT=35.0 (±0.09) μM; IC_{50} LDH= 32.5(±0.04) μM untuk 72 jam. Aktiviti sitotoksiti goniothalamin pada sel HepG2 telah

juga dipastikan menggunakan pengujian pewarna biru Trypan. Goniothalamin telah mengurangkan bilangan sel hidup (tidak berwarna) yang berhubung dengan pertambahan bilangan sel mati (berwarna) dan indek viabiliti pada pengukuran IC_{50} adalah 52 ± 1.73% bagi sel HepG2 dan 62 ± 4.36 % untuk sel Chang selepas 72 jam. Sel-sel yang didedahkan pada goniothalamin pada kepekatan terendah (2.3 μM), IC_{50} (keputusan MTT), dan kepekatan tertinggi (150 μM) pada 24, 48 atau 72 jam dan kemudian diperiksa kesan pada kitaran sel (menggunakan aliran sitometrik) atau pertumbuhan sel (menggunakan pengujian BrdU ELISA). Aktiviti sitotoksik goniothalamin adalah berkait dengan perencatan sintesis DNA, seperti yang ditunjukkan oleh pengurangan penggabungan BrdU. Pada 72 jam terakhir untuk goniothalamin berkepekatan 2.3 μM, peningkatan sel normal hati Chang kekal pada 97.6% berbanding pertumbuhan sel kawalan, sementara sel kanser hati HepG2 telah menurun kepada 19.8% berbanding pertumbuhan sel kawalan. Goniothalamin menyebabkan pengumpulan sel apoptotik hipodiploid pada kitar sel yang dianalisis menggunakan aliran sitometri. Goniothalamin menghentikan sel HepG2 dan sel Chang pada fasa G2/M pada darjah yang berbeza. Pemeriksaan melalui mikroskop cahaya pada sel HepG2 dan sel Chang yang terdedah terhadap goniothalamin pada kepekatan yang berbeza hingga 72 jam menunjukkan perubahan pada morfologi sel; i.e. sel membulat dan diikuti dengan kehilangan sifat pelekatan antara sel seterusnya menghasilkan sel yang kecut dan mengerut. Tambahan pula, sel apoptotik adalah lebih banyak dalam sel HepG2 yang dirawat dengan goniothalamin (84 ± 4.58%) untuk 72 jam berbanding sel-sel yang tidak dirawat (4 ± 2.65%) yang diukur dengan teknik warnaan TUNEL. Melalui kajian toksisiti goniothalamin, jenis kematian sel iaitu apoptosis atau nekrosis perlu dinilai. Oleh itu, pewarnaan dengan fluoresen yang dilabelkan dengan annexin V dengan

gabungan propidium iodida telah dilakukan pada sel HepG2 dan sel Chang yang terdedah pada goniotahlamin. Imbasan pancaran laser sitometri propidium iodida dan annexin V pada sel yang diwarnakan telah menunjukkan bahawa perencatan pertumbuhan akibat goniothalamin adalah konsisten dengan aruhan kuat proses apoptosis pada peringkat akhir disebabkan oleh kehilangan intergriti membran, maka sel-sel tersebut telah mempamerkan peningkatan bacaan dwi-positif untuk annexin V dan propidium iodida sehingga 85.87 ± 0.78 dan 57.69 ± 1.12 untuk sel HepG2 dan sel Chang masing-masing selepas 24 jam. Dalam menastikan mekanisma apoptotik bagi sel yang dirawat dengan goniothalamin, pengukuran aktiviti caspase 3 telah dijalankan dengan keadaan ujikaji yang sama. Keputusan ujikaji menunjukkan aktiviti caspase 3 adalah meningkat awal dengan signifikan dalam sel Chang yang dirawat IC_{50} (574% berbanding kawalan) iaitu selepas 24 jam dan akhir pada sel HepG2 yang dirawat IC_{50} iaitu selepas 72 jam (879 % berbanding kawalan). Hasil kajian ini mencadangkan suatu mekanisma yang mungkin untuk perencatan kuat pertumbuhan akibat goniothalamin pada sel cancer hati (HepG2) dengan sensitiviti yang rendah pada sel asas hati normal Chang terhadap bahan ini. Suatu ciri penting sitotoksisiti goniothalamin adalah pengantaraannya adalah melalui proses apoptosis.

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Thank you.

I certify that a Thesis Examination Committee has met on 12 October 2009 to conduct the final examination of Mothanna Sadiq Obaid Al-Qubaisi on his thesis entitled "Cytotoxicity of Goniothalamin on the Human Hepatocellular Carcinoma HepG2 Cell Line" in accordance with the 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 Master of Science.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Sciences.

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Date: 11 February 2010

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.

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MOTHANNA AL-QUBAISI

Date: 3 March 2010

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LIST OF ABBREVIATIONS

Abrivation	Full name
AFB1	Aflatoxin B1
ATCC	The American Type Culture Collection
BrdU	Bromodeoxyuridine
Chang cells	Normal liver cell line
CO_2	Carbon Dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EtOH	Ethanol
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
G_0	Resting phase
G_1	Gap between mitosis and DNA synthesis
G_2	Gap between DNA synthesis and mitosis
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCl	Hydrochloric acid
HepG2	Human hepatocellular liver carcinoma cell line
HRP	Horseradish Peroxidase

IC_{50}	Inhibition concentration at 50 percent
ICAM-1	Inter-Cellular Adhesion Molecule 1
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
LDH	Lactate Dehydrogenase
LDL	Low-density lipoproteins
M	Mitosis
mL	Mililiter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NaHPO4	Disodium hydrogen phosphate anhydrous
NaOH	Sodium Hydroxide
Nm	Nanometer
PBS	Phosphate buffer saline
pH	Minus the decimal logarithm of the hydrogen ion activity in an aqueous solution
PI	Propidium iodide
PS	Phosphatidyl serine
RB 1	Retinoblastoma protein 1
S	DNA synthesis
SI	Selective Index
STAT	Signal Transducers and Activators of Transcription
TDT	Deoxynucleotidyl Transferase
TP53	Tumor protein p53
TUNEL	TdT-mediated dUTP Nick End Labeling

VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoproteins
WNT	Proteins have roles in embryogenesis, cancer and in normal physiological processes
µg	Microgram

CHAPTER I

INTRODUCTION

Goniothalamus macrophyllus (locally named "Gajah beranak") is used traditionally as health tonic during pregnancy and to treat cold as well as fever (Burkill, 1953). The screening of this plant for bioactive compounds has resulted in the isolation of a large number of cytotoxic compounds, notably styryl-lactone derivatives, acetogenins, aporphine alkaloids and related alkaloids (Blasquez *et al*., 1999). These compounds have also been found to possess strong antimicrobial (Khan *et al*., 1999), larvicidal (Ee, 1998), antimalarial (Likhitwitayawuid *et al*., 1997) and embryotoxic activities (Sam *et al*., 1987).

Goniothalamin is a styryl-lactone compound isolated from the root and stem of *Goniothalamus macrophyllus* (Sam *et al*., 1987). Cytotoxicity of goniothalamin was reported in a number of carcinoma cell types isolated from a variety of tissues such as colon cancer cell line (Ângelo *et al*., 2005), breast cancer cell lines (Chen *et al*., 2005) and lung carcinoma (Chatchai *et al*., 2005). Skin fibroblast, human fibroblast and bovine kidney are normal cell lines that showed resistant to this compound (Chatchai *et al*., 2005).

More than 80% of Hepatocellular carcinoma HCC cases occur in the Far East and Southeast Asia. Although immunization has been successful against hepatitis B virus (HBV), a changing disease burden of HCC has been observed in many parts of the

world because of the increasing prevalence and duration of hepatitis C virus (HCV) infection in these countries (Kao and Chen, 2005).

Hepatocellular carcinoma (HCC) is refractory to chemotherapy because of tumor heterogeneity and the development of multidrug resistance phenotypes (Huang *et al*., 1992; Legoix *et al*., 1999). The Hepatocellular Carcinoma HCC cells are presenting mutations of p53 (transcription factor works as a tumor suppressor that is involved in preventing cancer), which lead to more aggressive resistance to chemotherapy (Heinze *et al*., 1999)

Doxorubicin is the best systemic chemotherapy with a variety of agents, including, epirubicin, mitoxantrone, cisplatin, and etoposide, either alone or in combination (Shah *et al*., 1998). It is often used in patients with HCC disseminated beyond the liver, although the response rates are generally of the order of only 15 %. In addition to that, doxorubicin is expensive and has serious side effects such as nausea, vomiting, mucositis, ulceration, necrosis of the colon and acute myeloid leukemia with a preleukemic phase and may cause heart failure (British Medical Association and Royal Pharmaceutical Society of Great Britain RPSGB, 2006).

Plant bioactive compounds have fewer side effects with low-cost when used in chemotherapy. Thus, the gearing of compounds, extracted from plants, for medicinal purposes becomes a workable thing. Based on this, the objectives of the study are:

1. To assess toxicity and selectivity of goniothalamin against Hepatocellular Carcinoma HepG2 cell line in comparison with normal liver (Chang) cell line.
2. To determine the mechanism of cytotoxicity, the treated cells with goniothalamin, have behaved.