UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC PROPERTIES OF ANTHRAQUINONES (NORDAMNACANTHAL AND DAMNACANTHAL) FROM ROOTS OF MORINDA ELLIPTICA

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By

LATIFAH SAIFUL YAZAN

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

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DEDICATION

I just want to voice out my "syukur" that beyond all question, after all, I managed to complete my PhD. Thank God for bestowing my life with the best and noblest people, my parents, **SAIFUL YAZAN JALALUDDIN** and **MARDIAH SURDA**. They are just as glorious as the sun that brings sunshine to my sullen sky. The persons who comfort me when I was crying like a baby, who always there for me the moment I felt just on my last legs, who told me again and again that I can do it, who told me not to lose heart, to take no account of what people say, and who forced me to carry on and carry out, and finally call forth this thesis. To my beloved husband, **YUSUFF ABDUL LATIF**, thank you for being a bosom, intimate friend of mine, to be all ears to my problems, to lend a hand when I have a hard row to hoe, and stand up for me whenever necessary. Thank God for giving me the opportunity to be a mother of two sons, **AIMAN RIDHWAN** and **ADIB RASYDAN**. The process of getting PhD showered me with fountain of experience. I learnt to take the bitter with the sweet, to take the rough with the smooth. Taking straight from the shoulder, I didn't have any intention to be head and shoulder over anybody or to compete an old hand or to get across anybody. I'm still at learning age that I need guidance to know the ropes. Do forgive me if there is a slip of the tongue. Let bygones be bygones and we have to move on because life goes on even if we don't like the way it is.

"Happiness lies for those who cry, those who hurt, those who have searched and those who have tried. For only they can appreciate the importance of people who have touched their lives"

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

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Chairman: Associate Professor Raha Abdul Rahim, Ph.D.

Faculty: Food Science and Biotechnology

The study on the cytotoxic properties of nordamnacanthal and damnacanthal, the anthraquinones isolated from the roots of *Morinda elliptica* (family Rubiaceae) were carried out on several cancerous cell lines including CEM-SS (T-lymphoblastic leukaemia), KU812F (chronic myelogeneous leukaemia), WEHI-3 (leukaemia), HT29 (colon cancer) and HeLa (cervical adenocarcinoma). The degree of cytotoxicity of the compounds were defined by their abilities at certain concentration to cause 50% reduction in cell number relative to the untreated sample, and termed as IC_{50} value. CEM-SS was observed to be the most sensitive cell line towards nordamnacanthal and damnacanthal with the IC_{50} values of 1.7 μg/ml and 10 μg/ml, respectively, as detected by the colorimetric tetrazolium-based assay (MTT). The compounds also showed cytotoxicity to the non-cancerous cell lines such as HF19 (lung fibroblast), human peripheral blood mononuclear (PBMC), 3T3 (mouse embryo) and Vero (monkey kidney fibroblast) but at very high concentrations (>30 μg/ml). The

microscopic analysis on the treated CEM-SS cells including light microscopy without staining or following staining with haematology polychrome, Giemsa and Wright's stains, fluorescence microscopy following staining with acridine orange and propidium iodide, and scanning and transmission electron microscopy showed that these compounds induced two types of cell death, apoptosis and necrosis.

At the molecular level, these compounds caused internucleosomal DNA cleavage producing multiple of 180-200 bp fragments that are visible as a "ladder" on the agarose gel. The DNA fragmentation has been found to be due the activation of the Mg^{2+}/Ca^{2+}-dependent endonuclease. The induction of apoptosis by nordamnacanthal was different from the one induced by damnacanthal in a way that it occurs independently of ongoing transcription process. Nevertheless, in both cases, the process of dephosphorylation of protein phosphates 1 and 2A, the ongoing protein synthesis and the elevations of the cytosolic Ca^{2+} concentration were not needed for apoptosis to take place. Nordamnacanthal and damnacanthal at their IC_{50} values showed different mechanism by which they exert their cytotoxic effects. Nordamnacanthal was found to have cytotoxic effect by inducing apoptosis in CEM-SS cells. Damnacanthal, on the other hand, showed cytostatic effect by causing arrest at the G0/G1 phase of the cell cycle. Nordamnacanthal was also found to reduce the expression of *bcl-2*, thus stimulating the process of apoptosis in CEM-SS cells.

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

CIRI-CIRI SITOTOKSIK ANTRAKUINON (NORDAMNACANTHAL DAN DAMNACANTHAL) DARIPADA AKAR *MORINDA ELLIPTICA*

Oleh

LATIFAH SAIFUL YAZAN

April 2003

Pengerusi: Profesor Madya Raha Abdul Rahim, Ph.D.

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Kajian ke atas ciri-ciri sitotoksik nordamnacanthal dan damnacanthal, antrakuinon

terhadap beberapa jujukan sel kanser seperti CEM-SS (T-limfoblastik leukemia), KU812F (kronik myelogeneous leukemia), WEHI-3 (leukemia), HT29 (kanser kolon) dan HeLa (adenokarsinoma serviks). Darjah kesitotoksikan sebatian-sebatian tersebut dinyatakan sebagai kebolehan mereka pada kepekatan tertentu yang menyebabkan pengurangan bilangan sel sebanyak 50% berbanding sampel yang tidak dirawat (kawalan), yang diistilahkan sebagai nilai IC_{50}. CEM-SS merupakan jujukan sel yang paling sensitif terhadap nordamnacanthal dan damnacanthal dengan nilai IC_{50} masing-masing, 1.7 μg/ml dan 10 μg/ml, seperti yang dikesan menggunakan kaedah kolorimetri berasaskan tetrazolium (MTT). Sebatian-sebatian tersebut juga menunjukkan kesan sitotoksik terhadap jujukan sel bukan kanser yang digunakan di dalam kajian ini seperti HF19 (fibroblas paru-paru), human peripheral blood mononuclear (PBMC), 3T3 (embrio tikus) dan Vero (fibroblas ginjal monyet), tetapi

pada kepekatan yang amat tinggi (>30 μg/ml). Analisa mikroskopi terhadap sel-sel CEM-SS yang dirawat dengan sebatian-sebatian tersebut menggunakan mikroskop cahaya, tanpa atau selepas pewarnaan dengan pewarna hemotologi polikrom iaitu Giemsa dan Stain's, mikroskopi floresen selepas pewarnaan dengan akridin oren dan propidium iodida, dan mikroskopi elektron imbasan dan transmisi menunjukkan mereka menyebabkan dua jenis kematian iaitu apoptosis dan nekrosis.

Pada peringkat molekul, sebatian-sebatian tersebut menyebabkan belahan di antara nukleosom pada DNA yang menghasilkan pecahan-pecahan bersaiz 180-200 bp yang kelihatan seperti "tangga" pada gel agaros. Belahan DNA tersebut didapati disebabkan oleh pengaktifan aktiviti endonuklease bergantung-Mg^{2+}/Ca^{2+}. Induksi apoptosis oleh nordamnacanthal berbeza dari yang disebabkan oleh damnacanthal di mana ia berlaku tanpa bergantung kepada proses transkripsi yang berterusan. Walau bagaimanapun, di dalam kedua-dua kes, proses defosforilasi protein 1 dan 2A, sintesis protein yang berterusan serta peningkatan pada kepekatan Ca^{2+} sitosol tidak diperlukan untuk apoptosis berlaku. Pada nilai IC_{50}, nordamnacanthal dan damnacanthal didapati mempunyai mekanisma yang berbeza dalam menunjukkan kesan sitotoksik masing-masing. Nordamnacanthal didapati mempunyai kesan sitotoksik dengan merangsang apoptosis dalam sel-sel CEM-SS. Sebaliknya, damnacanthal menunjukkan kesan sitostatik melalui penahanan pada fasa G0/G1 dalam kitaran sel. Nordamnacantal juga didapati mengurangkan penzahiran gen *bcl-2*, lalu merangsang proses apoptosis di dalam sel-sel CEM-SS.

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CHAPTER 1

INTRODUCTION

The incidence of cancer is increasing dramatically in the last decade world-wide. The numbers representing the patients suffering from this cruel disorder continuously increased each year. In the United States, for instance, cancer is the second leading cause of death with estimated new cancer cases and deaths of 1,284,900 and 555,500, respectively, for 2002 (Cancer Facts & Figures-2002, 2002). In Malaysia, even though there are no thorough and detailed statistical studies being done yet on the incidence of cancer, 35,000 new cases have been reported registered yearly, making it the fourth major cause of death in the country (National Cancer Registry of Malaysia, 2002).

Cancer results from the uncontrolled growth and spread of abnormal cells. In addition to inherited genetic mutations and other biological factors, environmental influences such as chemicals and radiation (including sunlight) can increase the risk of having cancer. These factors may act individually or together. The disorder takes two forms of abnormal growth of tissue. One is benign and grows slowly. Its main distinction is that, once formed, it stays where it is. In all cancers, malignant tumours sometimes grow rapidly, and they spread, affecting not only healthy tissues nearby but also often invading vital parts of the body, such as the lungs, breasts and stomach. Cancer cells break away from the original growth and travel in the blood stream.

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They may also, at the same time, invade the lymphatic system that defends human body against bacterial invasion (Evans, 1991).

As cancer is considered a systemic disease due to its metastatic properties, therefore, the cure from cancer will likely come from some type of systemic treatment. Chemotherapy could be such as systemic therapy for cancer (Verweij and de Jonge, 2000). Excellent results of chemotherapy have been obtained in a small range of cancers such as lymphoma and leukaemia (Garrett and Workman, 1999). Therefore, there is still a long way to go to achieve the necessary jump in the long-term survival and curability of the major solid cancers especially those with metastatic forms.

Comparatively, chemotherapy, the use of drugs to treat cancer, is a relatively young strategy to those two treatment modalities (surgery and radiotherapy). The steadily increasing interest in the development of drugs against cancer particularly started since the late 1950s (Verweij and de Jonge, 2000). In general, anticancer drugs destroy cancer cells by stopping them from growing or multiplying at one or more points in their life cycle. They interfere with cell replication to cause either tumour cell killing (cytotoxic drugs) or cessation of growth (cytostatic drugs) (Wilkes, 1996). Even though chemotherapy can now be considered the main curative treatment, drugs for cancer are not only becoming impersonal but also expensive and highly toxic. They may be genotoxic, teratogenic and fetotoxic. In certain cases, the

drugs may increase the risk of developing a second cancer (secondary primary) (BIOMED*4090, 2002).

As a consequence, based on the fact that human survival has always depended on plants whereby the early man relied entirely on them for food and medicine, scientist are making a move into searching for more effective and less harmful drugs to treat cancer from nature. Indeed, there are thousands of interesting and important medicines from nature.

Natural products especially higher plants have historically served and remain as templates for the development of many important classes of drugs such as mitotic inhibitor and antibiotic. The World Health Organization (WHO) has estimated that at least 80 per cent of the world's population relies mainly, if not totally, on natural medicines. Even in industrialized countries, up to 40 per cent of all pharmaceuticals are derived from natural sources (Polunin and Robbins, 1992). The therapeutic properties of plants were subjected to continuous assessment and evaluation not only recently but over thousands of generations. Through previous efforts, plants have become a viable source of clinically useful anticancer agents such as vincaleukoblastine (vinblastine; Velban®) and leurocristine (vincristine; Oncovin®) from *Catharanthus roseus* (Neuss and Neuss, 1990), and taxol (paclitaxel; Taxol®) from *Taxus brevifolia* (Wani *et al.*, 1971).

Currently, Malaysian tropical rain forests that contain a disproportionate share of the earth's plants with interesting pharmacologically active constituents provide a possible 'new avenue' with the emergence of a potent antitumour compound, a styrylpyrone derivative (SPD) that has been extracted from a plant from a family of Annonaceae (Azimahtol Hawariah, 1999). It has been predicted to be another potential therapy for breast cancer besides the commercially available hormonal drug, tamoxifen.

Cancer occurs when cells become abnormal and keep dividing and forming cells without control or order. The health of all multicellular organisms, including humans, depends not only on the ability to produce new cells but also on the ability of individual cells to self-destruct when they become superfluous or disordered, through a process called apoptosis or programmed cell death. The fact that some cells are behaving aberrantly, which eventually lead to the development of various disorders, particularly cancer, are due to the failure of the cells to undergo apoptosis (Reed and Tomaselli, 2000).

Apoptosis (in classical Greek means "dropping off") is indeed an ongoing process and it is as fundamental to cellular and tissue physiology as are cell division and differentiation due to its pivotal role in normal organ development, deletion of vestigial structures in embryogenesis, control of cell numbers and elimination of nonfunctional, harmful, abnormal or misplaced cells, as well as in many genetic and acquired diseases (Granville *et al.*, 1998). Cells experiencing apoptosis give