UNIVERSITI PUTRA MALAYSIA

CYTOTOXICITY OF MAHANIMBINE, MURRYAFOLINE A AND S-BENZYLDITHIOCARBAZATE ON HUMAN LEUKEMIC CELL LINE, CEM-SS

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Chairman: **Professor Abdul Manaf Ali, Ph.D.**

Faculty: **Food Science and Biotechnology**

Mahanimbine, a carbazole alkaloid was isolated from an ether extract of the stem bark of *Murraya koenigii* whilst Murrayafoline A was isolated from petroleum ether extract of the roots of *Murraya koenigii*. *S*-Benzyldithiocarbazate is a dithiocarbazic acid Schiff base derived from S-alkyl esters. They were found to exhibit cytotoxic activity against CEM-SS human T-lymphoblastic leukemic cells. The cytotoxic activity of Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate that inhibit 50 % growth (IC_{50}) of CEM-SS were 6 μg/ml, 5 μg/ml and 7.5 μg/ml respectively. For comparative purposes, the IC_{50} of several commercial cytotoxic drugs against CEM-SS were determined. The inhibition effect of Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate were better than Methotrexate (IC_{50} > 30 μg/ml), Doxorubicine (IC_{50} = 21 μg/ml), Cytarabine (IC_{50} > 30 μg/ml) and Colchecine (IC_{50} = 8 μg/ml).

These compounds were found to be less active than cis-diamine dichloroplatinum and Vinorelbine tartrate with a IC_{50} value of 3 μg/ml. In contrast, these three compounds were found to be less active against normal mouse fibroblasts cell, 3T3 with the IC_{50} value of 11 μg/ml (Mahanimbine), 17 μg/ml (Murrayafoline A) and 10 μg/ml (*S*-Benzyldithiocarbazate) respectively. The study showed that the proliferation of cells was inhibited before the cells were being killed. In addition, Mahanimbine, MurrayafolineA and *S*-Benzyldithiocarbazate caused programmed cell death by showing apoptotic features such as nucleus fragmentation, cell shrinkage, membrane blebbing and formation of apoptotic bodies. These were further confirmed with DNA laddering in agarose gel electrophoresis assay due to DNA fragmentation. DNA laddering was obtained after 24 hours of treatment by these three compounds in a dose-independent but time-dependent way. Mahanimbine and Murrayafoline A were shown to arrest CEM-SS cells at G_1 phase of cell cycle using flowcytometry method. As a result, Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate were found as potent antitumor agents.

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

SITOTOKSIKSITI MAHANIMBINE, MURRAYAFOLINE A AND *S*-BENZYLDITHIOCARBAZATE KE ATAST JUJUKAN SEL LEUKEMIK MANUSIA, CEM-SS

Oleh

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Pengerusi: **Profesor Abdul Manaf Ali, Ph.D.**

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Mahanimbine, sejenis karbazole alkaloid yang diasingkan dari ekstrak eter daripada batang kulit kayu *Murraya koenigii* manakala Murrayafoline A diasingkan dari ekstrak petrolium eter daripada akar *Murraya koenigii*. *S*-Benzyldithiocarbazate pula adalah asid dithiocarbazik Schiff bas yang dihasil daripada ester S-alkyl. Mereka didapati menunjukkan akitiviti sitotoksik ke atas jujukan sel T-lymphoblastik leukemik manusia, CEM-SS. Aktiviti sitotoksik Mahanimbine, Murrayafoline A dan *S*-Benzyldithiocarbazate yang dapat merencatkan pertumbuhan sel CEM-SS sebanyak 50 peratus (IC_{50}) adalah 6 μg/ml, 5 μg/ml and 7.5 μg/ml masing-masing. Untuk tujuan perbandingan, IC_{50} bagi beberapa jenis dadah sitotoksik komersial ke atas CEM-SS juga ditentukan. Kesan perencatan Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate adalah lebih baik daripada Methotrexate (IC_{50} > 30 μg/ml),

Doxorubicine (IC_{50} = 21 μg/ml), Cytarabine (IC_{50} > 30 μg/ml) dan Colchicine (IC_{50} = 8 μg/ml). Sebatian-sebatian ini adalah kurang aktif berbanding dengan cis-diamine dichloroplatinum dan Vinorelbine tartrate dengan nilai IC_{50} 8 μg/ml. Sebaliknya, ketiga-tiga sebatian ini didapati kurang aktif ke atas jujukan sel fibroblast tikus yang normal, 3T3 dengan nilai IC_{50} 11 μg/ml (Mahanimbine), 17 μg/ml (Murrayafoline A) dan 10 μg/ml (*S*-Benzyldithiocarbazate) masing-masing. Kajian ini telah menunjukkan bahawa pertumbuhan sel-sel akan direncatkan sebelum mereka terbunuh. Tambahan lagi, Mahanimbine, Murrayafoline A dan *S*-Benzyldithiocarbazate mengarahkan kematian sel terprogram dengan menunjukkan sifat apoptotik seperti penyerpihan nukleus, pengecutan sel, membran membengkak dan pembentukkan badan apoptotik. Penentuan secara lebih lanjut telah ditunjukkan dengan pembentukkan "tangga DNA" dalam elektrophoresis gel agarose terhasil dari penyerpihan DNA. Tangga DNA didapati selepas rawatan 24 jam oleh ketiga-tiga sebatian secara bergantung kepada masa rawatan dan bukan dos. Mahanimbine and Murrayafoline A dapat menahan CEM-SS sel pada fasa G_1 dengan menggunakan flowsitometri. Justeru itu, Mahanimbine, Murrayafoline A dan *S*-Benzyldithiocarbazate didapati berpotensi sebagai agen antibarah.

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LIST OF ABBREVIATIONS

%	percentage
AO	acridine orange
ATCC	American Type Culture Collection
bp	base pairs
EDTA	Ehylenediamine Tetraacetic Acid
IC_{50}	Inhibition concentration at 50%
IR	Infra-red
mg	milligram
ml	milliliter
mM	millimolar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
nm	nanometer
O.D.	Optical Density
OsO_4	osmium tetraoxide
PBS	phosphate buffered saline
PI	propidium iodide
rpm	rotation per minute
SEM	scanning electron microscope
TEM	transmission electron microscope
UV	ultraviolet
µg	microgram

CHAPTER 1

INTRODUCTION

Cancer is now the public's most feared disease. The global incidence of cancer is soaring due to rapidly aging populations in most countries. Over the next 25 years there will be a dramatic increase in the number of people developing cancer. Globally, 10 million new cancer patients are diagnosed each year and this will surge to 20 million by the year 2020 (Sikora, 1999). American Cancer Society reported that since 1990, approximately 12 million new cancer cases have been diagnosed. In the states, about 1,268,100 Americans are expected to die of cancer in the year of 2001- more than 3000 people a day. This increment is fuelled in part by the globalization of unhealthy lifestyles. Billions of dollars are spent annually, on cancer research by the drug industry, but a cure for cancer appears illusive.

Comprehensive articles on areas as wide-ranging as chemoprevention, cancer genetics and human cancer therapy have stimulated great interest. An understanding of the varied mechanisms leading to cancer has favored the development of many new cancer therapies, most notably perhaps the emergence of curative cytotoxic drug treatments of some previously uniformly fatal forms of cancers. Dramatic technological change is likely in surgery, radiotherapy and chemotherapy to increase cure rates, but at a price.

Decades of modern treatments have taught us that cancer is a "smart" disease and surgery has become conservative due to technological improvements. Therefore, the role of chemotherapy has become more defined to beat "smart" cancer cells via smarter therapies. This is a considerable challenge. It is matched, however by the real sense of excitement that has developed in the field of new anticancer drug discovery (Baring, 1997). This excitement is based on the premise that we can design much smarter and more effective therapies by aiming to counteract or exploit the very genetic and biochemical abnormalities that drive the disease itself (Workman, 1994). The treatments of the next century will be rationally designed, less toxic and more effective.

The third millenium is upon us and achievements in cancer research had reached to a stage where tremendous technological advances for cancer are available to millions of patients suffering from and succumbing to unconquered neoplastic diseases. Besides that, there is enormous potential for the discovery of innovative cancer drugs with imporved efficacy and selectivity for this millenium. The world is in a health transition and cancer drug discovery is re-inventing itself, in order to exploit the latest intellectual technological developments. Hopefully in the next 25 years, will be a time of unprecedented change in the way in which we will control cancer.

There are four major phases in drug discovery, target identification and validation, lead identification, lead optimization and clinical drug candidate and in this study, the very early stage of drug discovery was being emphasized. Two natural products Mahanimbine, Murrayafoline A isolated from *Murraya koenigii* (curry leaf)

whose leaves are widely used in local cooking and a synthetic compound *S*-Benzyldithiocarbazate were being approached and studied in order to discover their enormous potential as chemotherapeutic drugs on T-lymphoblastic leukemia.

The objectives of this study are:

i) to determine the cytotoxicity of Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate on human T-lymphoblastic leukemic, CEM-SS cell.

ii) To study the effect of Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate on CEM-SS cells in terms of proliferation, morphological changes and the mode of cell death induced by the compounds.

iii) To identify the mode of action of Mahanimbine, Murrayafoline A and *S*-Benzyldithiocarbazate.

CHAPTER 2

LITERATURE REVIEW

2.1 Traditional Medicine

Traditional medicine using herbal exists in every part of the world. In the history of medicine, the necessity of helping sick people goes back to the origin of mankind. In many places of the world special ways of medical treatment were found. By looking back into history, we found that nearly all cultures from ancient times until the present day have been using plants as a source for medicine. Generally, we can distinguish three major areas of traditional medicine: Chinese, Indian and European and the art of practicing herbal medicine stretches back over more than thousand of years (Vogel, 1991). However, there are a few basic difficulties that traditional medicine not accepted worldwide. The Health Authorities of this individual country mostly accept the experiences gathered in one cultural area but not by another Health Authority elsewhere. Another basic difficulty is the fact that traditional medicine usually consists of many herbal or animal or inorganic ingredients. There is a wide variation in the quantity of pharmacological active substances in each plant. Differences in methods of extraction and purification influence the results. Besides that, it is quite often traditional recipes lacked of sufficient quality control and drug standardization. (Vogel, 1991). Therefore, it's important to internationalize some standardize extracts from these herbal drugs by going through a required developing procedures from Health Authority.

2.2 Family Rutaceae

This family comprises trees, shrubs and climbers recognized from their resinous, lime-like smell of the broken twigs, fruits or of the crushed leaves. Many essential oils such as citronella and bergamot are extracted by distillation from plants of this family and mostly of these oils are used in native medicine (Kirthika and Basu, 1935).

This family consists of 161 genera and 1700 species throughout the world and found mostly in warm countries (Mabberly, 1987). In Malaysia, there are 16 genera and 50 species and mostly are found in lowland area.

2.2.1 Genus Murraya

Genus Murraya is from Angiosperma's division, Dycotilidon's Class, Sepindales's order, Rutaceae's (Lemon) family (Heywood, 1979) and Aurantiodea's subfamili. Genus Murraya was named after John Andrew Murray (1740-1791), a Sweedish botanist. Among the seven species from this genus, only *Murraya paniculata* has aromatic flowers (Huxley, 1992). *Murraya koenigii* (curry leaf) is one of the most widely used plants whose leaves are added to curries to improves flavor. It is usually cultivated for its aromatic smell, which are used as natural flavorings in curries and sources (Sastri, 1952).

Various parts of *M. koenigii* have many medicinal uses and values as raw material for traditional medicine and popular in India (Kirthikar and basu, 1935). It have been

reported that the leaves and the roots can cure piles and heat removal of the body, inflammation and itching, while the powdered leaf is used to aid healing of fresh cuts. The green leaves could be eaten raw for the treatment of dysentery (Drury, 1978). Barks and roots are used to relieve skin eruptions and bites by poisonous animals (Dasturs, 1970). Currently, this plant has been used in the industry, where fresh leaves are steam distilled under reduced pressure to yield volatile oils (curry leaf oil) which can be used as a fixative for a heavy type of soap perfume (Joseph *et al.*,1985). Biological activities of Malaysia plants were first reported by Nakashini *et al.* in 1965. *Murraya koenigii* has been proven to be a rich source of carbazole alkaloids. Isolation work on the stem bark carried out by Reish *et al.* 1994 gave a new non-cyclized compound that postulated as biogenetic precursors of mahanimbine.

2.2.2 *Murraya koenigii* and its uses

Murraya koenigii is a member of the large Rutaceae family and represented by about 161 genera and 1700 species. In Malaysia, nearly 60 species of this family can be found including two species of *Murraya*: namely *M. koenigii* and *M. panicluata.*

M. koenigii (curry leaf) is one of the most widely used plants whose leaves are added to curries to imporves flavour. This intensive pungent, aromatic leaves are best when fresh, but adequately retain their potency for some time after picking.

2.3 Cancer

Cancer is a genetic disease, because it can be traced to alterations within specific genes, but in most cases, it is not an inherited disease. The genetic alterations that lead to most cancers arise in the DNA of a somatic cell during the lifetime of the affected individual. Because of these genetic changes, cancer cells proliferate uncontrollably, producing malignant tumors that invade surrounding healthy tissue (Karp, 1999).

Because of its impact on human health and the hope that a cure might be developed, cancer has been the focus of a massive research effort for decades. Cancer researchers have been working for many years to develop more effective and less debilitating treatments. Current treatments, such as chemotherapy and radiation, lack the specificity needed to kill cancer cells without simultaneously damaging normal cell, as evidenced by the serious side effects that accompany these treatments (Karp, 1999).

2.3.1 The Biology of Cancer

At the cellular level, the most important characteristic of a cancer cell is its loss of growth control. When malignant cells or so-called neoplasm in the scientific or medical term are cultured on a culture dish under a limited growth factor condition, they continue to grow, pilling on top of one another to form clumps (Albert, 1989). Malignant cells escape from many of the normal homeostatic mechanisms that control proliferation (Goodman

1994). Transformation of normal cells to neoplasm cells may be triggered by several factors, including chemical exposure, viruses and radiation. This showed that malignant cells are not responsive to the types of regulatory signals that cause their normal counterparts to cease growth and division. Uncontrolled growth combined with the tendency to become metastatic makes malignant cells the deadly threat that they are.

2.3.2 Classification of Tumors

Generally there are two kinds of tumors benign and malignant. Benign tumor is a tumor composed of cells that are no longer responsive to normal growth controls, but that lack the capability to invade normal tissues or metastasize to distant sites whereas malignant tumors often spread to neighboring tissues and even other parts of the body (Albert, 1989).

2.3.3 The Genetics of Cancer

Whenever cells of cancerous tumor are genetically scrutinized, they are invariable found to have arisen from a single cell. Cancer results from the uncontrolled proliferation of a single wayward cell (cancer is said to be monoclonal). Malignant transformation requires more than a single genetic alteration. The development of a malignant tumor is a multistep process characterized by a progression of genetic alterations in a single line of cells that makes the cells increasingly less responsive to the body's normal regulatory machinery and able to invade normal tissues (Karp, 1999).