UNIVERSITI PUTRA MALAYSIA

BIOLOGICAL ACTIVITIES AND MOLECULAR ANALYSIS OF NOVEL DITHIOCARBAZATE COMPLEX COMPOUNDS ON GLIOMA CELL LINES

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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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Chairman: **Associate Professor. Rozita Rosli, PhD**

Faculty: **Medicine and Health Sciences**

The object of research in the exploration of new chemotherapy agents is to kill cancerous cells and not harm the healthy cells. In addition, an effective dose of these agents is essential in conducting clinical studies in the treatment of cancer. In this study, an investigation of the anticancer effects of a group of synthetic compounds on human glioma cell lines was carried out. Initially, 11 compounds were screened using cytotoxicity assays. The most active compounds were found to be derived from bis (S-methyl-β-*N*-(2-acetylfuran) dithiocarbazate) (SMDB) and bis (S-benzyl-β-*N*-(2-acetylfuran) dithiocarbazate) (SBD4) complexed with zinc, cadmium and platinum ions. The glioma cell lines, A172, U87MG and T98G and normal brain cell line HCN-2, were used in this study. The IC_{50} values of the cell lines treated with the compounds were determined by using (3-4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium

bromide (MTT) assay. Tamoxifen was used as a control as it is the current drug of choice in the treatment of brain cancer.

From the cytotoxicity assays, it was found that the compounds which showed the most potential are SMDB-Cd and SMDB-Zn. The IC_{50} values for SMDB-Cd on A172, U87MG, T98G and HCN-2 were 0.65µg/ml, 0.29µg/ml, 0.4µg/ml, and 1.4µg/ml, while that for SMDB-Zn were at 3.7µg/ml, 1.76µg/ml, 2.7µg/ml and 7µg/ml, respectively. The IC_{50} values for tamoxifen for the same cell lines were 6.7µg/ml, 5.3µg/ml, 6.3µg/ml and 6µg/ml respectively.

Several methods were employed towards understanding the mechanism of action at the molecular level for SMDB-Cd and SMDB-Zn on glioma cell lines. Tunel assay displayed the typical morphological features of apoptosis cells with condensed and fragmented nuclei at 48 hours. The percentage of apoptotic cells in all treated cells with tamoxifen, SMDB-Zn and SMDB-Cd were significantly ($p<0.05$) increased.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used in monitoring the gene expression level of two key genes, Epidermal Growth Factor Receptor (EGFR) and Mouse Double Minute 2 (MDM2). The expression of EGFR gene was suppressed in all three-cell lines. However, MDM2 gene was suppressed only in A172 and T98G. Therefore, the suppression of EGFR and MDM2 by the compounds was one of the pathways to apoptosis in the glioma cells.

In the flow cytometry analysis, the effect of SMDB-Cd and tamoxifen on the cell cycle after 3, 6, 12 and 24 hr treatment showed glioma cells A172, U87MG and T98G were arrested in G_1 phase and the SMDB-Zn arrested glioma cell lines U87MG, T98G and A172 in, G2/M, S phase and G_1 phase, respectively.
The SMDB-Cd and tamoxifen arrested the cell cycle by preventing replication (phase specific G_1) whereas SMDB-Zn was not phase specific which can arrest the cell at any point in the cell cycle.

Results, of caspase-8/9 activity assay of tamoxifen, SMDB-Cd and SMDB-Zn on glioma cells showed that caspase-8 activity was significantly induced but no significant activity for caspase-9 was observed. Therefore, the activation of caspase-8 may be the mechanism through which tamoxifen, SMDB-Cd and SMDB-Zn induces apoptosis.

The comet assay used to study the genotoxic activity of SMDB-Cd and SMDB-Zn in CHO cell line showed no genotoxic activity in both compounds. In conclusion, the two compounds have the potential to be developed as chemotherapeutic agents.

Nilai IC_{50} untuk sel-sel tersebut yang telah dirawat dengan sebatian-sebatian di-atas dipastikan dengan menggunakan kaedah (3-4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT). Tamoksifen telah digunakan sebagai kawalan memandangkan ia adalah dadah pilihan semasa dalam rawatan kanser otak.

Dari kaedah sitotoksik itu sebatian-sebatian yang ditemui menunjukkan potensi adalah SMDB-Cd dan SMDB-Zn. Nilai IC_{50} untuk SMDB-Cd pada A172, U87MG, T98G dan HCN-2 adalah 0.65µg/ml, 0.29µg/ml, 0.4µg/ml, dan 1.4µg/ml, sementara itu bagi SMDB-Zn adalah 3.7µg/ml, 1.76µg/ml, 2.7µg/ml and 7µg/ml. Nilai IC_{50} bagi tamoksifen pula untuk sel-sel yang sama tersebut adalah 6.7µg/ml, 5.3µg/ml, 6.3µg/ml and 6µg/ml.

Beberapa kaedah telah dijalankan ke arah memahami mekanisme tindakan SMDB-Cd dan SMDB-Zn tersebut dalam sel-sel glioma pada peringkat molekul. Kaedah Tunel telah menunjukkan ciri-ciri morfologi yang tipikal bagi sel-sel apoptotik dengan nukleusnya yang menjadi padat dan pecah pada 48 jam, peratus sel-sel yang apoptotik dalam semua sel-sel yang dirawat bersama tamoxifen, SMDB-Zn dan SMDB-Cd adalah sangat bermakna ($p<0.05$).

"Reverse Transcription-Polymerase Chain Reaction" (RT-PCR) telah digunakan dalam pemerhatian paras ekspresi gen terhadap dua gen ini, "Epidermal Growth Factor Receptor" (EGFR) dan Mouse Double Minute 2 (MDM2). Ekspresi gen EGFR telah dihalang didalam ketiga-tiga sel yang digunakan.

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I am very grateful to all my family members for their continued moral support of me in pursuing my dream. This work may not have masterialised without the prayers and love from my wife Rabiea Amor. I would like to dedicate this Ph.D, thesis to them and my committee.

I certify that an Examination Committee met on 13th December 2005 to conduct the final examination of Shaban A. Kh. Awidat on his Doctor of Philosophy thesis entitled "Biological Activities and Molecular Analysis of Novel Dithiocarbazate Complex Compounds of Glioma Cell Lines" in accordance with 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 follows:

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

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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.

SHABAN A. KH. AWIDAT

Date: 16/1/06

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BBB	Blood Brain Barrier
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CMT	Carrier-Mediated Transport
CFS	Cerebral Spinal Fluid
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
EGFR	Epidermal growth factor receptor
EDTA	Ethylenediamine tetraacetic acid
EMS	Ethyl methenesulfonate
MDM2	Murine double minute 2
PDGF	Platelet-Derived Growth Factor
pRB	Retinoblastoma protein
RMT	Receptor-Mediated Transport
RNA	Ribose nucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction

SBD4	(S-benzyl-β-*N*-(2-acetylfuran) dithiocarbazate)
SBD4-Cd	(S-benzyl-β-*N*-(2-acetylfuran) dithiocarbazate) Cd (II)
SBD4-Pt	(S-benzyl-β-*N*-(2-acetylfuran) dithiocarbazate) Pt (II)
SBD4-Zn	(S-benzyl-β-*N*-(2-acetylfuran) dithiocarbazate) Zn (II)
SMDB	S-methyl-β-*N*-(2-acetylfuran) dithiocarbazate
SMDB-Cd	(S-methyl-β-N-(2-acetylfuran)-dithiocarbazato) Cd (II).
SMDB-Pt	(S-methyl-β-*N*-(2-acetylfuran)-dithiocarbazato) Pt (II)
SMDB-Zn	(S-methyl-β-*N*-(2-acetylfuran)-dithiocarbazato) Zn (II)

CHAPTER 1

INTRODUCTION

1.1 General Background

Cancer is one of the three main causes of death among the economically active population. The two other main causes of mortality worldwide are accidents and cardiovascular diseases. Annually, there are more than 6 million deaths from a type of malignant neoplasia worldwide (Tovar-Guzman *et al.,* 2001). The number of new cancer cases has been increasing over the past nine decades. The Malaysian Ministry of Health (1995) reported that malignant neoplasm is the major cause of death in government hospitals (45%), which is 2.8 times higher than heart diseases (16%). A total 26,089 of cancers were diagnosed among all residents in Peninsular Malaysia in the year 2002, comprising 11,815 males and 14,274 females. An estimated 10,000 cases were however, not registered. In terms of risk, 1 in 5.5 Malaysians can be expected to get cancer in his/her lifetime. Taking into accounts unregistered cases, the risk would be 1 in 4 Malaysians. The crude rate for all cancers in the year 2002 was 118.9 per 100,000 males and 148.4 per 100,000 females. (Lim *et al.,* 2002).

1.2 Brain tumor

Primary tumors of the central nervous system account for less then 1.5% of all the cancer cases reported in the United States each year. According to the Malaysian national cancer registry in 2002, brain cancer incidence per 100,000 populations is 2.3 in males and 1.7 in female.

These infrequent tumors are the third leading cause of cancer-related deaths among men 15-54 years of age and fourth leading cause of death for women 15-34 years of age. Moreover, primary brain tumors are the most common solid tumor of childhood and the second leading cause of cancer death in children after leukemia (Kilic *et al.,* 2000).

Pathological approaches have shown that at the time of the initial diagnosis, most malignant glioma tumors have spread more then 15 mm over the area that can be identified by magnetic resonance imaging (MRI) scan (Kelly *et al.,* 1987, Greene *et al.,* 1989). Several molecular mechanisms are involved in the development of gliomas and their progression to more malignant tumors. For instance, the progression to higher grade glioma is associated with inactivation of the p53 tumor suppression gene on chromosome 17p, as well as over-expression of platelet-derived growth factor (PDGF) (Dunn *et al*., 2000). Furthermore, progression to glioblastoma involves amplification of epidermal growth factor receptor (EGFR), murine double minute 2 (MDM2) genes and the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (Schlegel *et al*., 1994, Biernat*, et al.,* 1997).

The prognosis for people affected by these rapidly growing tumors is currently very poor. Therapeutic modalities for malignant gliomas, including surgery, radiation and chemotherapy are of limited effectiveness and novel treatment modalities must be explored.

A direct targeting of cytotoxicity agents to cancer cells represents a modern approach to the treatment of malignant glioma and other cancers, because it should improve tumor inhibition and decrease toxicity.

1.3 Nitrogen-sulphur Donor Ligands

Coordination compounds are molecules containing coordinate bonding where ligands have excess electrons and can form coordinate linkages upon interaction with metal centers. The ligands may be neutral molecules or ions. Ligands are attached to the central atom by dative bonds or coordinate covalent bonds. In an ordinary covalent bond, each of the bonded atoms contributes one electron to the electron pair that forms the bond. The coordinating atom or ligand, called the donor, donates a pair of electrons to the central atom, called the acceptor. Proceeding from the donor atom to the acceptor atom often depicts the bond. Interaction between metal ions and ligands results in the formation of complexes. The entire aggregate of central atom and ligands is generally called a complex.

The study of nitrogen-sulfur donor ligands continues to be of great interest to researchers. Dithiocarbazate, NH_2NHCS_2 and its substituted derivatives have received considerable attention over the past few decades (Tarafder and Roy, 1988). The chemistry of such ligands warrants further study because dithiocarbazic acid and the Schiff bases derived from its S-alkyl esters form an interesting series of ligands whose properties can be greatly modified by introducing organic substituents into the ligand molecules thereby inducing different stereochemistry in the resultant metal complexes.

The synthesis of the ligands and metal complexes are of interest because of the intriguing observation that different ligands show different biological properties, although they differ only slightly in their molecular structures (Ali *et al.*, 1987; Majumder *et al.,* 1988).

Transition metal complexes of these ligands are widely studied because of their potential for therapeutic use (Tarafder and Roy 1988; Ali *et al.,* 1996). For example, the Schiff base of 2-benzoylpyridine with S-methyldithiocarbazate (SMDTC) inhibits the growth of bacteria *Escherichia coli* and *Staphylococcus aureus* to some extent while that with S-benzyldithiocarbazate (SBDTC) shows no effect at all on the two mentioned bacteria (Gou *et al.,* 1990). The bioactivities of the ligands and the metal complexes such as cytotoxicity, antimicrobial and anticancer activities have not yet been widely studied. The mode of interaction of these compounds with the cancer cells and microbes are yet to be investigated.

In the present study, the anticancer effects of newly synthesized pure compounds on glioma cell lines, A172, U-87MG and T98G cell lines were investigated. Besides, screening these compounds as potential novel anti-cancer drugs for glioma, this study also investigates the mechanism involved, including the study on apoptosis, gene expression, cell cycle and genotoxic activity.

1.4 Significance of the present study

This project is of great importance due to the fact that some of the studied compounds are essentially new and no studies have been performed to investigate the action of these compounds toward cancer cells other than initial screening. It is anticipated that these compounds will open up many interesting avenues for further research. In other words, this project will give a new dimension to this field of brain cancer research and possibly by carrying out further studies on these new compounds, more information in this field will be gathered. This information may also enable further studies, for instance anticancer activities of related compounds. A number of compounds were tested (AR28, SB02 SPDRC, SMDCT, SBOTC, SMDB-Zn, SMDB-Pt, SBD4-Cd, SBD4-Pt, SBD4-Zn and SMDB-Cd) using cytotoxicity assay. SMDB-Cd and SMDB-Zn were found to be the most effective and hence were focused in this study. The Schiff bases and their metal complexes used in this study were synthesized and characterized by elemental analyses and various physico-chemical techniques (Chew *et al.*, 2004).

Objectives:

The objectives of this study were:

1. To study the cytotoxic effects of the pure synthetic compounds, (AR28, SB02 SPDRC, SMDCT, SBOTC, SMDB-Zn, SMDB-Pt, SBD4-Cd, SBD4-Pt, SBD4-Zn and SMDB-Cd) on glioma cell lines, A172, U-87MG and T98G.
2. To study the effect of the most effective compounds on the expression of EGFR and MDM2 genes.
3. To observe the morphology of the treated glioma cell lines by staining using fluorescence and light microscopy and to quantitatively determine apoptosis using tunnel assay.
4. To study the effect of the pure synthetic compounds on the cell cycle by using flow cytometry.
5. To study the mechanism of apoptosis induction by the compounds in glioma cells by determining caspase 8 and 9 activation.
6. To investigate the genotoxic effect of the compounds in CHO cells using comet assay and measuring the DNA damage using CASP software.