UNIVERSITI PUTRA MALAYSIA

MECHANISMS OF ANTITUMOUR ACTIVITY OF 3, 19-(2-BROMOBENZYLIDENE) ANDROGRAPHOLIDE (SRJ09)

LIM SIANG HUI

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IMPORTANT

The following manuscript " **MECHANISMS OF ANTITUMOUR ACTIVITY OF 3,19-(2-BROMOBENZYLIDENE)ANDROGRAPHOLIDE (SRJ09)**" is submitted to the School of Graduate Studies, Universiti Puta Malaysia, in fulfuilment of the requirements for the Degree of Master of Science by Lim Siang Hui. This manuscript can only be used for personal viewing and no part of this manuscript may be reprinted, linked to, or otherwise redistributed, in any form or by any means, without first obtaining the prior written consent of the author.

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MECHANISMS OF ANTITUMOUR ACTIVITY OF 3,19-(2-BROMOBENZYLIDENE)ANDROGRAPHOLIDE (SRJ09)

By

LIM SIANG HUI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

MECHANISMS OF ANTITUMOUR ACTIVITY OF 3,19-(2-BROMOBENZYLIDENE)ANDROGRAPHOLIDE (SRJ09)

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September 2007

Chairman: Associate Professor Johnson Stanslas, PhD

Faculty: Medicine and Health Sciences

To date, most of the clinical cytotoxic anticancer drugs target all rapidly dividing cells and are non-selective in their mechanism of action by disrupting essential components that are crucial to both malignant and normal cells. Hence, the search for more effective and selective anticancer drugs is currently being researched actively involving the various entities of the drug discovery programme. We at UPM have shown that andrographolide (AGP), a compound isolated from a local herb, *Andrographis paniculata,* to have anticancer activity *in vitro* and *in vivo*. In order to improve the antitumour properties of AGP, semi-synthetic derivatives of this compound were synthesised in our laboratory, with the aim of identifying the most promising anticancer compound among the AGP derivatives and to elucidate the mechanism(s) of action of the compound. The *in vitro* antitumour study showed that 3,19-(2-bromobenzylidene)andrographolide (SR09) displayed better antitumour activity when compared with AGP and other derivatives namely 3,19-(2-chlorobenzylidene)andrographolide (SRJ11) and 3,19-(3-chloro-4-fluorobenzylidene) andrographolide (SRJ23). The antitumour activity of AGP, SRJ09, SRJ11 and SRJ23 was shown to be not compromised by P-glycoprotein activities in MES-SA Dx5 multidrug resistant cell line. The time-course study

revealed SRJ09 had a rapid acting interval compared with AGP. SRJ09 was previously shown to induce G1-phase cell cycle arrest and in this study the effect was shown attributed to increased of p21 (CDK inhibitor) expression without affecting the expression of cyclin D1. Apoptosis was the main mode of cell death induced by SRJ09 and was p53 and bcl-2 independent, which might suggest that SRJ09 act through the extrinsic apoptotic pathway. A simple pharmacokinetic study was performed in Balb/c for the purpose of dose selection for *in vivo* study revealed that SRJ09 had a relatively short half-life but was able to reach *in vitro* cytotoxic concentration range. In a subsequent *in vivo* antitumour study, SRJ09 delayed quadruple tumour growth by 4 day in HCT-116 colon cancer xenografted mice treated with 400 mg/kg SRJ09 (q4d×3) when compared with control. In conclusion, SRJ09 have been proven as a lead anticancer agent given to its ability to induce *in vitro* cell cycle arrest and apoptosis and to have *in vivo* antitumour activity. Therefore, further studies in improving the anticancer properties of SRJ09 by chemical modification will be advantageous.

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

MEKANISME AKTIVITI ANTIKANSER 3,19-(2-BROMOBENZILEDINE)ANDROGRAPHOLIDE (SRJ09)

Oleh

LIM SIANG HUI

September 2007

Pengerusi: Profesor Madya Johnson Stanslas, PhD

Fakulti: Perubatan dan Sains Kesihatan

Sehingga kini, kebanyakan agen antikanser klinikal bertindak ke atas sel yang membahagi dengan cepat dan mempunyai mekanisme yang tidak selektif dengan mengganggu komponen asas yang penting untuk kedua-dua sel kanser dan sel normal. Oleh yang demikian, penemuan agen antikanser yang lebih efektif dan selektif kini dikaji dengan aktif dengan melibatkan pelbagai entiti program penemuan ubatan. Kajian kami di UPM menunjukkan bahawa andrographolide (AGP), sebatian yang dipencil dari *Andrographis paniculata*, mempunyai aktiviti antikanser secara *in vitro* and *in vivo*. Untuk meningkatkan potensi antitumor AGP, terbitan semisintetik sebatian ini disintesis di makmal kami, bertujuan untuk mengenalpasti sebatian antikanser yang poten di antara terbitan AGP dan seterusnya memahami mekanisme tindakannya. Secara *in vitro*, 3,19-(2-bromobenzylidene)andrographolide (SRJ09) menunjukkan kelebihan aktiviti antikanser berbanding dengan AGP dan terbitan yang lain seperti 3,19-(2-chlorobenzylidene)andrographolide (SRJ11) dan 3,19-(3-chloro-4-fluorobenzylidene)andrographolide (SRJ23). Aktiviti antikanser AGP, SRJ09, SRJ11 dan SRJ23 didapati tidak dipengaruhi oleh aktiviti P-glikoprotein sel MES-SA Dx5 yang resistan terhadap pelbagai agen antikanser. Kajian berlandaskan masa

menunjukkan SRJ09 mempunyai sela masa tindakan yang pantas berbanding dengan AGP. Kajian terdahulu menunjukkan bahawa SRJ09 merencat kitaran sel pada fasa G1 dan dalam kajian ini, kesan ini didapati berpunca dari peningkatan ekspresi p21 (perencat kinase bergantung siklin) dan tidak menpengaruhi ekspresi siklin D1. Kajian yang selanjutnya menunjukkan bahawa apoptosis merupakan punca utama kematian sel yang diaruh oleh SRJ09 dan adalah bebas daripada regulasi p53 dan Bcl-2. Untuk tujuan penentuan dos kajian *in vivo*, kajian farmakokinetik ringkas ke atas mencit Balb/c menunjukkan bahawa SRJ09 mempunyai separuh hayat yang pendek tetapi mampu mencapai julat konsentrasi sitotoksik *in vitro*. Dalam kajian *in vivo*, SRJ09 melewatkan pertumbuhan tumor kepada empat kali ganda selama empat hari dalam mencit yang dixenograf dengan kolon kanser HCT-116 yang dirawat dengan 400 mg/kg SRJ09 (q4d×3) berbanding dengan kawalan. Kesimpulannya, SRJ09 dikenalpasti sebagai agen antikanser pilihan utama kerana kebolehannya untuk merencat kitaran sel dan mengaruh apoptosis secara *in vitro* serta menunjukkan aktiviti antikanser secara *in vivo*. Justeru itu, kajian lanjutan untuk memperbaiki aktiviti antikansernya melalui modifikasi kimia adalah bermanfaat.

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I certify than an Examination Committee met on 19th September 2007 to conduct the final examination of Lim Siang Hui on his Master of Science thesis entitled "Mechanisms of Antitumour Activity of 3,19-(2-bromobenzylidene)andrographolide (SRJ09), a Semisynthetic Derivative of Andrographolide" 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:

Muhammad Nazrul Hakim Abdullah, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Asmah Rahmat, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohamad Aziz Dollah, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohd Zaini Asmawi, PhD
Professor
School of Pharmaceutical Sciences
Universiti Sains Malaysia
(External Examiner)

_____

Hasanah Mohd Ghazali, PhD
Professor/ Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This was thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Johnson Stanslas, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Mohammad Nazil Salleh, PhD
Lecturer
School of Life Sciences
Technology Park Malaysia Academy
(Member)

Zauyah Yusuf, PhD
Professor
Department of Medical Microbiology and Immunology
Universiti Kebangsaan Malaysia
(Member)

_____

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 January 2008

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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LIM SIANG HUI

Date:

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AGP	andrographolide
AIF	apoptosis-inducing factor
AO	acridine orange
APAF	apoptotic protease activating factor-1
ATP	adenosine triphosphate
AUC	area under curve
bp	base pair
C_0	initial concentration
Ca^{2+}	calcium ion
CDK	cyclin dependent kinase
Cl	clearance
C_{max}	maximum concentration
CO_2	carbon dioxide
DD	death domain
DISC	death-inducing signalling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOX	doxorubicin
DR	death receptors
dTMP	2'-deoxythymidine-5'-phosphate
dUMP	2’-deoxyuridine-5’-phosphate
ECGS	endothelial cell growth supplement

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	feotal bovine serum
FGF	fibroblast growth factors
Fig	figure
GI_{50}	50% growth inhibition concentration
HCl	hydrochloride acid
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HUVEC	human umbilical vein endothelial cell
i.p.	intraperitoneum
IGF	insulin-like growth factor
IgG	immunoglobulin type-G
K_{el}	elimination rate constant
LC_{50}	50% lethal concentration
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
$MgCl_2$	magnesium chloride
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NaCl	natrium chloride
NAD	nicotinamide adenine dinucleotide
NCI	National Cancer Institute
PAGE	polyacrylamide gel electrophoresis

PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PBS-T	PBS containing 0.1% Tween 20
PDGF	platelet-derived growth factor
PI	propidium iodide
RIP	receptor-interacting protein
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxidative species
RR	resistant ratio
RTV	relative tumour volume
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error mean
Smac	second mitochondria-derived activator of caspases
SOM	self-organise maps
T½	half-life
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	tumour growth factor
TGI	total growth inhibition
T_{max}	time to achieved C_{max}
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	tumour necrosis factor-related apoptosis inducing ligand
UV	ultraviolet

V	volt
V_d	volume of distribution
VEGF	vascular endothelial growth factor
w/v	weight over volume

CHAPTER 1

INTRODUCTION

1.3 Overview

Cancer is a new growth of tissue in which cell multiplication is uncontrolled and progressive. Unlike benign tumour cells, cancer cells exhibit the properties of invasion and metastasis and are highly anaplastic. In 2002, it is reported that cancer is the second major cause of death, which account 12.5% of total deaths worldwide (The World Health Report 2003). Surgical excision, radiation therapy and chemotherapy are the main approaches to treat cancer. Unlike surgery and radiation therapy, chemotherapy is considered a systemic treatment in which the drugs circulate in the blood circulations to eradicate cancer micrometastases at distant sites from the original cancer. Therefore, chemotherapy is often administered in conjunction with local therapy such as surgery and radiation to obtained optimal effects. The use of anticancer drugs as part of the treatment strategy for cancer has greatly improved the overall prognosis of cancer patients.

To date, most of the anticancer drugs target all rapidly dividing cells and are non-selective in their mechanism of action by disrupting essential components or metabolic pathways that are crucial to both malignant and normal cells (Ewesuedo and Ratain 2003). Therefore, scientists are still looking for more effective and type-selective antitumour drugs with minimum side effects. Several strategies were employed which include chemical modification or combinatorial chemistry involving

existing natural and synthetic products, structure-based drug design and new natural products discovery. The search for new natural products from the vast biodiversity is an importance source of structural diversity that yield unusual and unexpected lead structures which served as starting points for chemical modification to derive an optimal drug (Young 1999).

In the early 2000, andrographolide (AGP) a compound isolated from *Andrographis paniculata* received much attention as a candidate to be developed into anticancer agents. AGP was shown to inhibit tumour growth both *in vitro* and *in vivo* (Stanslas et al. 2001; Rajagopal et al. 2003). AGP was also reported to cause G_1 phase cell cycle arrest (Stanslas et al. 2001; Rajagopal et al. 2003) and apoptosis (Cheung et al. 2005; Kim et al. 2005; Zhou et al. 2006). Shortly, AGP was derivatised through various chemical modifications in an effort to improve its antitumour potential (Nanduri et al. 2004; Jada et al. 2006). The derivatives synthesised by coupling of two hydroxyl groups in AGP (C-3 and C-19) by reacting AGP with benzaldehydes having different functional groups are generally shown to have an improvement in terms of antitumour activity (Jada et al. 2006). Hence, in this study several lead derivatives were selected and evaluated, in order to identify the most potent anticancer compound among AGP derivatives and their mechanism(s) of action.