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IN VITRO GROWTH INHIBITION, MOLECULAR MECHANISMS OF CELL CYCLE ARREST, AND APOPTOSIS IN PROSTATE CANCER CELLS BY SRJ23

WONG HUI CHYN

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By

WONG HUI CHYN

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

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March 2013

Chairman : Associate Professor Johnson Stanslas, PhD

Faculty : Institute of Bioscience

SRJ23 (3, 19-(3-chloro-4-fluorobenzylidene andrographolide), a new semisynthetic derivative of andrographolide (AGP) was shown to exhibit selective anticancer activity against the hormone-independent prostate cancer cell lines (PC-3 and DU-145) in the USA National Cancer Institute (NCI) *in vitro* anticancer screen. Hence, in this study SRJ23 was investigated further for its mechanisms of cell cycle arrest and apoptosis. The microculture tetrazolium (MTT) assay was utilized in assessing the *in vitro* growth inhibition and cytotoxicity of SRJ23 and its parent compound, AGP against three human prostate cancer cell lines (PC-3, DU-145 and LNCaP). Subsequently, flow cytometry was used to analyse the cell cycle distribution of treated cells. Fluorescence microscopy was performed to determine the morphological cell death. DNA fragmentation and annexin V-FITC/PI flow cytometry analyses were carried out to confirm apoptosis induced by SRJ23. Quantitation of cell cycle and apoptotic regulatory proteins were determined by western blot analysis. SRJ23 was found to be more potent than AGP in exerting growth inhibition and cytotoxicity. The activity of the compound was selective

towards PC-3 cells and induced G_2/M arrest which led to predominantly apoptotic mode of cell death. The internucleosomal DNA fragmentation induced by SRJ23 was inhibited in the presence of caspase 8 inhibitor (Z-IETD-FMK). To induce G_2/M cell cycle arrest, the compound downregulated CDK1 without affecting the levels of CDK4 and cyclin D1. Interestingly, the compound induced G_1 cell cycle arrest in LNCaP and DU-145 cell lines, which attributed to increase expression of p21 and downregulation of CDK4 and cyclin D1 but without affecting the levels of CDK1. Additionally, SRJ23-treated LNCaP cells showed increased levels of wild-type p53. However, the compound did not affect mutant p53 level in DU-145 cells. AGP also induced G1 arrest in both the cell lines *via* increased the expressions of p21 and downregulated CDK4 but without affecting the levels of CDK1 and cyclin D1. However, unlike SRJ23, it did not affect the wild-type p53 protein in LNCaP cells. The induction of apoptosis by SRJ23 is associated with increased caspase 8 expression and activation. This thought to have induced cleavage of Bid into tBid. Additionally, expression and activation of executioner caspase 9 and pro-apoptotic Bax proteins, with a concomitant down-regulation of the anti-apoptotic Bcl-2 protein level were noted in all the three prostate cancer cell lines. AGP produced similar effects except it failed to affect Bcl-2 protein level in all three prostate cancer cell lines. Therefore, based on these findings, SRJ23 is proven to be more potent than AGP in inducing growth inhibition, cell cycle and apoptosis. The molecular events related to these effects have been established and undoubtedly points to the fact that some of pathways involved in SRJ23's anticancer effect differ compared with AGP. As such the former is being considered a lead compound in the discovery of clinical anti-prostate cancer agents.

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

PENAMBATAN PERTUMBUHAN DAN MOLEKULAR MEKANISMA PENYEKATAN KITARAN SEL, DAN APOPTOSIS SEL PROSTAT KANSER *IN VITRO* OLEH SRJ23

Oleh

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SRJ23 (3, 19-(3-kloro-4-florobenzilidin andrografolida) adalah agen semisintetik terbaru terbitan andrografolida (AGP). Berpandukan ujian saringan in vitro dari Institut Kanser Kebangsaan Amerika Syarikat, sebatian ini menunjukkan aktiviti antikanser terpilih terhadap sel kanser prostat jenis tidak bersandar hormon (PC-3 dan DU-145). Justeru, kajian ini menjurus kepada penyelidikan tentang mekanisma penyekat kitaran sel dan apoptosis oleh SRJ23. Asai mikrokultur tetrazolium (MTT) *in vitro* telah digunakan untuk mengkaji kesitotoksikan dan perencatan pertumbuhan ke atas 3 jenis sel kanser prostat (PC-3, DU-145 dan LNCaP) oleh AGP dan terbitannya, SRJ23. Seterusnya, analisa sitometri aliran digunakan untuk menganalisis edaran kitaran sel terhadap sel yang telah dirawat dengan sebatian tersebut. Pendarfluor mikroskopi juga digunakan untuk menentukan morfologi sel mati. Bagi mengesahkan apoptosis, sejenis mod kematian sel, analisa fragmentasi DNA dan sitometri aliran annexin V-FITC/PI telah digunakan. Kuantifikasi protein yang terlibat dalam kitaran sel dan apoptosis telah ditentukan dengan menggunakan teknik ‘western blot’. SRJ23 telah didapati lebih sitotoksik dan berkesan dalam

merencatkan pertumbuhan sel kanser berbanding dengan AGP. SRJ23 adalah lebih selektif terhadap sel PC-3 dan menyebabkan sekatan kitaran sel pada fasa G_2/M. Ini seterusnya menyebabkan kematian sel tersebut akibat apoptosis. Tambahan lagi, dengan kehadiran penambat caspase 8 (Z-IETD-FMK), fragementasi DNA internukleusomal yang disebabkan oleh SRJ23 adalah terhalang. SRJ23 menyebabkan sekatan kitaran sel pada fasa G_2/M dengan merendahkan ekpresi protein CDK1 tanpa menjejaskan ekspresi CDK4 dan cyclin D1. Penemuan yang menarik juga didapati di mana SRJ23 menyebabkan sekatan kitaran sel pada fasa G_1 dalam sel LNCaP dan DU-145 dengan meningkatkan ekspresi protein p21 dan pada masa yang sama merendahkan ekspresi protein CDK4 dan cyclin D1, tanpa menjejaskan ekspresi CDK1. Tambahan lagi, sel LNCaP yang telah dirawat dengan SRJ23 menunjukkan peningkatan ekspresi protein p53 jenis-liar. Walau bagaimanapun, sebatian tersebut tidak menjejaskan ekspresi mutan p53 dalam sel DU-145. AGP juga menyebabkan sekatan kitaran sel pada G_1 di dalam kedua-dua sel melalui peningkatan ekspresi p21 dan merendahkan ekspresi CDK4, tetapi tanpa menjejaskan ekspresi CDK1 dan cyclin D1. Walaubagaimanapun, tidak seperti SRJ23; AGP tidak menjejaskan ekspresi protein p53 jenis-liar dalam sel LNCaP. Induksi apoptosis oleh SRJ23 telah dikaitkan dengan peningkatan ekpresi dan pengaktifan caspase 8. Ini menyebabkan penukaran daripada Bid kepada tBid. Pada masa yang sama, ekspresi dan pengaktifan pelaksana caspase 9 dan protein pro-apoptotik Bax, serta penurunan ekspresi protein anti-apoptotik Bcl-2 juga dikesan di dalam ketiga-tiga sel kanser prostat tersebut. AGP memberi kesan yang sama tetapi ia tidak menjejaskan ekspresi protein Bcl-2 di dalam ketiga-tiga jenis sel kanser prostat tersebut. Justeru itu, berdasarkan penemuan dalam kajian ini, dapat disimpulkan bahawa SRJ23 telah terbukti lebih berkesan daripada AGP dalam

menyebabkan perencatan pertumbuhan, sekatan kitaran sel dan apoptosis. Mekanisma molekular sebatian ini yang menyebabkan kesan ini telah ditemui dan tanpa sangsi telah dibuktikan bahawa beberapa tapak laluan yang melibatkan kesan antikanser SRJ23 adalah berbeza berbanding dengan AGP. Oleh yang demikian, SRJ23 dianggap sebagai sebatian pendahulu dalam penemuan agen anti-kanser klinikal untuk rawatan kanser prostat.

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I certify that a Thesis Examination Committee has met on the 26th of March 2013 to conduct the final examination of Wong Hui Chyn on her thesis entitled "***In vitro* growth inhibition, molecular mechanisms of cell cycle arrest, and apoptosis in prostate cancer cells by SRJ23**" 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 (with Thesis) degree in Pharmacology.

Members of the Examination Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

WONG HUI CHYN

Date: 26 March 2013

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AGP	Andrographolide
AIF	Apoptosis-inducing factor
ANOVA	Analysis of variance
AO	Acridine orange
AP	*Andrographis paniculata*
APAF	Apoptotic protease activating factor
APC	ATP binding cassette
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid
AUC	Area under concentration-time curve
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BHC	Benzene hexachloride
BID	BH3 interacting-domain death agonist
BNC	Hexachlorocyclohexane
BP	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CDK	cyclin-dependent kinase

CI	Clearance
CKI	Cyclin-dependent kinase inhibition
Cmax	Maximum concentration
CO_2	Carbon dioxide
COMPARE	Computerized pattern recognition algorithm
COX	Cyclooxygenase
CYP	Cytochrome P450
DISC	Death-inducing signalling complex
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DOX	Doxorubicin
DR	Death receptor
DTP	Developmental Therapeutics Program
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal regulated kinase
FADD	Fas-associated protein death domain
FASL	Fas ligand
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GCCP	Good cell culture practice
GI50	50% growth inhibition concentration
GSH	Glutathione
GST	Glutathione S-trasnferase
GTP	Glutamyl transferase

HCC	Hepatocellular carcinoma
HCl	Hydrochloride acid
HIV	Human immunodeficiency virus
HR	Hour
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
Kel	Elimination rate constant
LC_{50}	50% lethal concentration
MAC-1	Macrophage adhesion molecule-1
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2
MDR	Multidrug resistance
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase
MRP	Multidrug resistance-associated protein
MTD	Maximum tolerated dose
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide hydrogenase
NCI	National Cancer Institute

NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS containing 0.1% Tween 20
PES	Polyethersulfone
P-gp	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol 12-myristate 13-acetate
pRb	Retinoblastoma protein
PS	Phosphatidylserine
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTX	Paclitaxel
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RR	Resistance ratio
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate

SOM	Self-organizing map
SPSS	Statistical Package for Social Sciences
SRJ09	3, 19-(2-bromobenzylidene) andrographolide
SRJ23	3, 19-(3-chloro-4-fluorobenzylidene andrographolide
STZ	Streptozotocin
T½	Half-life
TCM	Traditional Chinese Medicine
TEMED	N, N, N', N'-tetramethylethylenediamine
TGI	Total growth inhibition
TIMP	Tissue inhibitor of metalloproteinase
Tmax	Time to achieved Cmax
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
TOP	Topoisomerase
TRAIL	TNF-related apoptosis-inducing ligand
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
w/v	Weight over volume
V	Volt
Vd	Volume of distribution
VEGF	Vascular endothelial growth factor
VIN	Vinblastine
ɣ-GTP	Gamma glutamyl-transpeptidase
Z-IETD-FMK	Caspase 8 inhibitor

CHAPTER 1

INTRODUCTION

1.1 Overview

Prostate cancer is the most frequently diagnosed age-related malignancy in men and has become a major health issue, being the second leading cause of cancer-related deaths in men worldwide after lung cancer (Jemal *et al*., 2010). Factors that determine the risk of developing prostate cancer are unfamiliar. Different studies based on epidemiological and genetic analyses demonstrate that age and hereditary factors are associated with an increase in the incidence of prostate cancer (Porkka and Visakorpi, 2004). Exogenous factors, such as diet, may also have an important impact on this risk (Aus *et al*., 2005).

During the past decade, the survival rate for advanced stages of prostate cancer has not improved despite significant advances in prostate cancer treatment (DeLancey *et al*., 2008). The high mortality rate observed in advanced prostate cancer patients is due to loss of androgen dependency for cancer cell growth that results in resistance to androgen ablation therapy (Cho *et al*., 2003). In addition, standard treatment options for localised prostate cancer, such as, surgical, radiation and hormone therapy, are associated with morbidities that often impair patient's quality of life, such as urinary incontinence and sexual dysfunction (Eton and Lepore, 2002).

Despite the large number of studies, scientific knowledge of the molecular mechanisms underlying the disease is still limited and poorly understood. This has become the biggest stumbling block in the discovery of new effective treatments for this deadly disease. Therefore, there is an urgent need to explore all possible avenues in finding effective therapeutic agents, be they of synthetic or natural origin.

Andrographolide (AGP) is a natural compound isolated from *Andrographis paniculata* (Matsuda *et al*., 1994). It is endowed with an interesting pharmacophore that displayed various pharmacological activities, such as, anti-inflammatory (inhibition of inducible nitric oxide (iNOS) expression) (Chiou *et al*., 1998), Mac-1 expression, and ROS production (Shen *et al*., 2000), anticancer (Stanslas *et al*., 2001) and hepatoprotective (Kapril *et al*., 1993).

Research on the anticancer potential of AGP has been one of the major focuses at the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia (UPM) for almost a decade, beginning with the discovery of activity of the compound against human breast cancer tumours in an animal model (Stanslas *et al*., 2001). Subsequently, there was a report of its potential in combating colon cancer (Rajagopal *et al*., 2003). AGP was also reported to have cytotoxic activity against various cancer cell lines representing different tumour types (Jada et al., 2007; Manikam and Stanslas, 2009) but lacked tumour selectivity (Jada *et al*., 2007). In addition, this compound demonstrated the ability to induce G_1 cell cycle arrest through induction of p27 and decreased expression of cyclin-dependent kinase (CDK) 4 in human tumour cell lines (Rajagopal *et al*., 2003).

Recent findings revealed AGP had anti-metastatic (Shi *et al*., 2009) and anti-angiogenic potential by decreasing MMP (matrix metalloproteinase) -7 expression in human colorectal carcinoma Lovo cells (Sheeja *et al*., 2007) and VEGF (vascular endothelial growth factor) expression in cancer cells (Zhao *et al*., 2008), respectively.

Another study by Pratheeshkumar *et al*. (2011) demonstrated using human umbilical vein endothelial (HUVEC) cells that the administration of AGP significantly retarded endothelial cell proliferation, migration and invasion as well as tube formation and inhibits *in vitro* angiogenesis by inhibiting MMP-2 and MMP-9 by regulating the nuclear translocation of transcription factors, such as p65, p50, c-Rel subunits of nuclear factor-κB, c-fos, activated transcription factor-2 and cyclic adenosine monophosphate (cAMP) response element-binding protein.

The effect of AGP on apoptosis is controversial. AGP is capable of protecting immune cells (thymocytes) or endothelial cells against apoptosis (Chen *et al*., 2004; Burgos *et al*., 2005). On the other hand, other reports suggest that AGP could induce apoptosis by activating the caspase cascade and by regulating Bcl-2 family proteins in human tumour cells (Kim *et al*., 2005; Zhou *et al*., 2006). In human leukaemic HL-60 cells, induction of apoptosis was linked with leakage of mitochondrial cytochrome *c* and upregulation of Bax expression (Cheung *et al*., 2005). The compound also displayed cell differentiation and apoptosis in acute promyelocytic leukaemia cells (Manikam and Stanslas, 2009). However, the detailed molecular mechanisms of AGP-induced apoptosis are still largely unknown and being actively studied by various groups (Shi *et al*., 2009; Yang *et al*., 2009; Chao *et al*, 2010; Lee

et al., 2010a; Lee *et al.*, 2010b; Parichatikanond *et al*., 2010; Yang *et al*., 2010; *Zhou et al*., 2010; Lee *et al*., 2011; Lin *et al*., 2011).

The elucidation of the mechanism of action of AGP remains inexplicable although many models have been proposed to illustrate the antitumour properties of AGP. However, the most intriguing finding on the mechanism of antitumour activity of AGP came from a study by Liang *et al*. (2008), who revealed this compound had a novel mechanism through its ability to promote degradation of the oncoprotein v-Src by attenuation of the Erk1/2 signalling pathway. A more recent finding by Tan *et al*. (2010) points to AGP's ability to exert its antitumour activity by affecting the receptor trafficking in cancer cells. The above proposed mechanisms are supported in part by the discovery that AGP predicted to have novel mechanism of action through the analysis of the USA National Cancer Institute (NCI)'s *in silico* self-organising map (SOM) (Jada *et al*., 2007).

Due to the immense studies reported on the pharmacological properties of AGP, in particular its anticancer activity, attempts to improve its antitumour potential was performed by derivatising AGP through various chemical modifications (Nanduri *et al*., 2004; Jada *et al*., 2006). The UPM group produced derivatives of AGP by coupling the two hydroxyl groups at C-3 and C-19 in the presence of various substituted benzaldehydes; an improvement in the antitumour activity of the derivatives was observed (Jada *et al*., 2008).

The biological activity of AGP derivatives revealed their ability to inhibit the growth of breast and colon cancer cells *in vitro* through the induction of cell cycle arrest and

apoptosis as well as displayed enhanced antitumour activities in comparison with the parent compound (Jada *et al*., 2008). One of the derivatives termed SRJ23 (3, 19-(3-chloro-4-fluorobenzylidene andrographolide) showed potent and selective growth inhibitory effect against prostate cancer cells in the NCI *in vitro* screen (Jada *et al*., 2008). However, the exact mechanism of the anti-prostate cancer activity of SRJ23 is still largely unknown. Therefore, in the present study, investigation of the *in vitro* growth inhibitory properties and examination of the mechanisms of cell cycle arrest and apoptosis induced by SRJ23 were performed in a panel of prostate cancer cells.

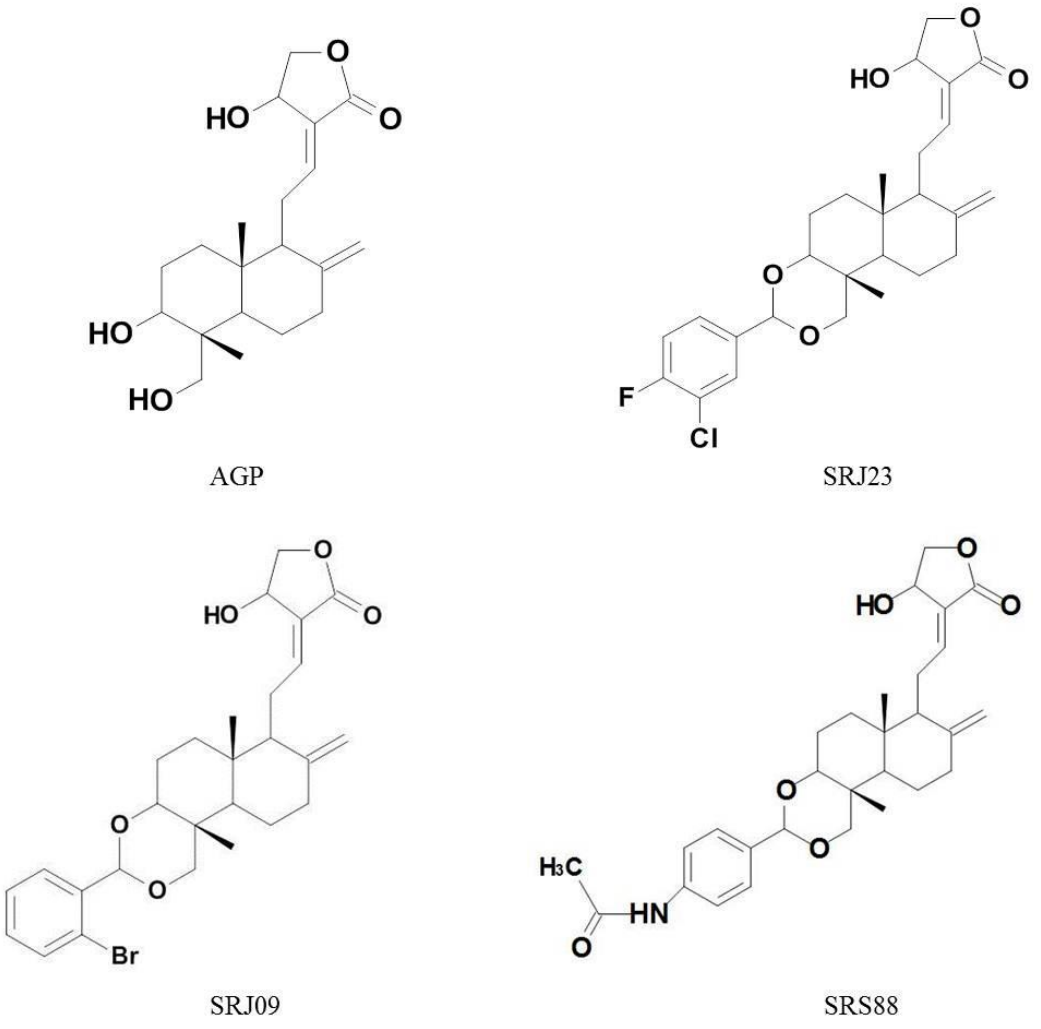

Figure 1.1: Chemical structures of AGP and its derivatives.

1.2 Hypothesis

SRJ23 is able to inhibit growth of prostate cancer cell lines by inducing cell cycle arrest and apoptosis through modulation of key proteins involved in growth and survival.

1.3 Objectives

The general objective of this study was to determine the *in vitro* anti-prostate cancer potential of SRJ23. Therefore, the study was undertaken with the following specific objectives:

1. To determine the *in vitro* growth inhibitory effects of SRJ23 on a panel of prostate cancer cell lines.
2. To determine the cell cycle arrest induced by SRJ23.
3. To investigate the mode of cell death induced by SRJ23.
4. To determine the mechanisms of cell cycle arrest induced by SRJ23 by evaluating the expression of key cell cycle mediators.
5. To determine the mechanisms of apoptosis induced by SRJ23 by evaluating the expression of key apoptotic mediators.

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LIST OF PUBLICATIONS

Publications

1. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Nordin Haji Lajis, and Johnson Stanslas. 2012. SRJ23, a potent inducer of cell cycle arrest and apoptosis in prostate cancer cells. *FASEB J*. 26: 851.9

2. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Nordin Haji Lajis, Seng Cheong Loke, Johnson Stanslas. 2011. Andrographolide induces cell cycle arrest and apoptosis in PC-3 prostate cancer cells. *African Journal of Pharmacy and Pharmacology* 5(2): 225-233.

3. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Nordin Haji Lajis, Johnson Stanslas. 2010. SRJ23, A new investigational anti-prostate cancer agent. *FASEB Journal* 24: 964.6

Posters

1. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Nordin Haji Lajis, Johnson Stanslas. 2012. SRJ23, a potent inducer of cell cycle arrest and apoptosis in prostate cancer cells. American Society for Pharmacology and Experimental Therapeutics (ASPET) Experimental Biology (EB) Meeting 2012. San Diego Convention Center, San Diego, California (Poster 773.1). 21st – 25th April 2012.

2. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Nordin Haji Lajis, Johnson Stanslas. 2010. SRJ23, a new investigational anti-prostate cancer agent. American Society for Pharmacology and Experimental Therapeutics (ASPET) Experimental Biology (EB) Meeting 2010. Anaheim Convention Center, Anaheim, California, Los Angeles (Poster 964.6). 24th – 28th April 2010

3. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Nordin Hj Lajis, Johnson Stanslas. A new semisynthetic compound derived from andrographolide displays impressive *in vitro* activity against prostate cancer cells. Conference on the Recent Development in Chinese Herbal Medicine. Nanyang Executive Centre (NEC), Nanyang Technological University (NTU), Singapore (Poster 35). 25th-26th January 2010.

4. **Wong Hui Chyn**, Sreenivasa Rao Sagineedu, Shaik Ibrahim Khalivulla, Nordin Hj Lajis, Johnson Stanslas. SRJ23, a promising new anti-prostate cancer agent. Pameran Reka Cipta, Penyelidikan dan Inovasi (PRPI) 2009. Dewan Besar (PKKSSAAS), Universiti Putra Malaysia (UPM) (Poster B-HE-IBS-205). 28th-30th July 2009.

5. **Hui Chyn Wong**, Sreenivasa Rao Sagineedu, Shaik Ibrahim Khalivulla, Nordin Hj Lajis, Johnson Stanslas. SRJ23, a promising new anti-prostate cancer agent. 23rd Malaysian Society of Physiology and Pharmacology Scientific Meeting 2009. Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur, Malaysia (Poster 61). 12th-13th May 2009.

IMPORTANT

The following manuscript "***IN VITRO* GROWTH INHIBITION, MOLECULAR MECHANISMS OF CELL CYCLE ARREST, AND APOPTOSIS IN PROSTATE CANCER CELLS BY SRJ23**" is submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the requirements for the Degree of Master of Science by Wong Hui Chyn. 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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