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SEMISYNTHESIS OF ANDROGRAPHOLIDE DERIVATIVES AND EVALUATION OF THEIR ANTITUMOUR PROPERTIES

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SEMISYNTHESIS OF ANDROGRAPHOLID DERIVATIVES AND EVALUATION OF THEIR ANTITUMOUR PROPERTIES

By

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Previously, andrographolide, which is the major diterpenoid of *Andrographis paniculata*, was shown to have *in vivo* antitumour activity against human breast tumour xenografts. In this study, among the four compounds isolated from *A. paniculata*, andrographolide was the most potent compound with a mean IC$_{50}$ value of 8 μM in MCF-7 human breast cancer cells. Neoandrographolide showed a weak cytotoxic effect, whereas 14-deoxy-11,12-didehydroandrographolide and 14-deoxyandrographolide failed to exhibit growth inhibitory effect at the highest tested concentration of 100 μM. Owing to this, andrographolide was considered as the lead compound in the discovery of potent and selective antitumour agents.

Using andrographolide isolated from *A. paniculata* as one of the starting materials, 3,19-
benzylidene andrographolide and 3,19-alkylidene andrographolide derivatives were synthesised by coupling of the two -OH groups present at C-3 and C-19 of andrographolide with different benzaldehydes and alkyl aldehydes, respectively. In addition, new derivatives were also synthesised by acetylation, oxidation, Heck and esterolysis reactions. The structures of new derivatives of andrographolide derivatives were confirmed by spectral analysis (\(^1\)H/\(^{13}\)C NMR, MS, FT-IR, UV).

Forty seven compounds including andrographolide were tested for antitumour activities in MCF-7 and HCT-116 (colon) cancer cell lines. Using a 72 h MTT cell viability assay, parameters of dose-response effects, GI\(_{50}\), TGI and LC\(_{50}\) were determined. The derivatives had submicromolar GI\(_{50}\) values, except for 3,19-(4-nitrobenzylidene)andrographolide (SRJ58), which showed the most potent activity with a GI\(_{50}\) value of 0.7 \(\mu\)M in MCF-7 cells. Only (Z)-2-[1-benzylamino-2-(5,5,6,8a-tetramethyl-2-methylene-decahydro-naphthalen-1-yl)-ethyl]-4-hydroxy-but-2-enoic acid benzylamide] (SRJ18), displayed a pronounced selectivity (approximately 8-fold) towards HCT-116 cells at the GI\(_{50}\) value compared with MCF-7 cells.

Out of the five compounds (3,19-isopropylideneandrographolide (SRJ01), 14-acetylandrographolide (SRJ03), 3,19-(2-bromobenzylidene)-14-deoxy-11,12-didehydroandrographolide (SRJ05), 3,19-(2-bromobenzylidene)andrographolide (SRJ09) and 3,19-(3,4-dimethoxybenzylidene)andrographolide (SRJ13)) tested against the 60 National Cancer Institute (NCI) of USA human cancer cell lines, only SRJ09 showed some form of selectivity towards cancers of the colon, central nervous system, renal and melanoma. The mechanism(s) of actions of the compounds were also studied by
determining their effect in inducing cell cycle arrest and apoptosis. Andrographolide, 
SRJ01 and SRJ03 induced G₁ and G₂/M arrest in MCF-7 cells, whereas 3,19-(4-
bromobenzylidene)andrographolide (SRJ08), SRJ09, 3,19-(3-bromobenzylidene) 
andrographolide (SRJ10), 3,19-(3-chloro-4-fluorobenzylidene)andrographolide (SRJ23) 
and 3,19-(2-fluorobenzylidene)andrographolide (SRJ27) induced only G₁-phase arrest 
in MCF-7 cells. SRJ09 down-regulated CDK4 (a G₁-phase regulator) protein levels in 
MCF-7 cells, which explains the G₁-phase arrest by the compound. NCI’s COMPARE 
mechanistic analysis revealed that the compounds antitumour activities were not similar 
to that of standard anticancer drugs with known mechanisms of action. Projection of 
SRJ03 in the Self-Organising Maps (SOMs) analyses of NCI suggested that this 
compound may be targeting cell cycle related phosphatases or kinases. However, 
andrographolide, SRJ01, SRJ05, SRJ09 and SRJ13 did not project in the known 
mechanism categories.

The mode(s) of cell death induced by SRJ09 and SRJ23, identified by fluorescence 
microscopy and flow cytometry, was confirmed to be apoptosis in HCT-116 cells.

In conclusion, novel derivatives of andrographolide, especially SRJ09, SRJ18 and 
SRJ58 are potential lead molecules for future antitumour studies to discover prospective 
clinical candidates.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai syarat memenuhi keperluan untuk Ijazah Doktor Falsafah

SEMISINTESIS TERBITAN ANDROGRAPHOLIDE DAN PENILAIAN CIRI-CIRI ANTITUMORNYA

Oleh

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October 2004


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Andrographolide merupakan diterpenoid utama tumbuhan Andrographis paniculata dan kajian terdahulu menunjukkan andrographolide mempunyai aktiviti anti-tumor secara in vivo terhadap xenograf tumor payudara manusia. Dalam kajian ini, andrographolide merupakan sebatian yang paling poten diantara empat sebatian daripada A. paniculata, dengan nilai min IC₅₀ 8 μM dalam sel kanser payudara manusia MCF-7. Neoandrographolide mempamerkan kesan sitotoksik yang lemah, manakala 14-deoxy-11,12-didehydroandrographolide dan 14-deoxyandrographolide gagal menunjukkan kesan perencatan tumbsesaran apabila diuji pada kepekatan tertinggi iaitu 100 μM.

Justeru itu, andrographolide telah dipilih sebagai sebatian asas dalam usaha menghasilkan agen antitumor yang poten dan selektif berasaskan struktur rangka andrographolide.
Dengan menggunakan andrographolide yang diasingkan daripada *A. paniculata* sebagai bahan asas, 3,19-benzilidene andrographolide dan 3,19-alkilidene andrographolide disintesis dengan mengkupelkan dua kumpulan –OH pada kedudukan C-3 dan C-19 andrographolide masing-masing dengan benzaldehid dan alkil aldehid. Selain itu, terbitan andrographolide juga disintesis melalui proses asetilasi, oksidasi, tindakbalas Heck dan esterolisis. Struktur bagi terbitan baru andrographolide disahkan dengan menggunakan analisis spektral (*¹H/-¹³C NMR, MS, FT-IR, UV*).

Kesemua sebatian termasuk andrographolide diuji untuk menentukan antitumor terhadap kultur kanser payudara, MCF-7 dan kanser kolon, HCT-116. Dengan menggunakan asai viabiliti sel MTT selama 72 jam, nilai GI₅₀, TGI dan LC₅₀ ditentukan. Kesemua sebatian terbitan menunjukkan nilai GI₅₀ submikromolar terhadap kedua-dua jenis sel terutamanya 3,19-(4-nitrobenzylidene)andrographolide (SRJ58), yang menunjukkan aktiviti paling poten dengan nilai GI₅₀ pada 0.7 μM. Antara sebatian-sebatian tersebut, 8-kali ganda (Z)-2-[1-Benzylamino-2-(5,5,6,8a-tetramethyl-2-methylene-decahydro-naphthalen-1-yl)-ethyl]-4-hydroxy-but-2-enoic acid benzylamide (SRJ18), menunjukkan selektiviti terhadap sel HCT-116 dengan katara pada nilai GI₅₀ berbanding sel MCF-7.

Daripada lima sebatian (3,19-isopropylideneandrographolide (SRJ01), 14-acetylandrographolide (SRJ03), 3,19-(2-bromobenzylidene)-14-deoxy-11,12-didehydroandrographolide (SRJ05), 3,19-(2-bromobenzylidene)andrographolide (SRJ09) and 3,19-(3,4-dimethoxybenzylidene)andrographolide (SRJ13)) yang telah diuji ke atas 60 jenis sel kanser oleh National Cancer Institute (NCI), USA, hanya SRJ09 menunjukkan
selektiviti terhadap kanser sistem saraf pusat dan melanoma.

Andrographolide, **SRJ01** dan **SRJ03** didapati mengaruh perencatan fasa G\(_1\) dan G\(_{2/M}\) pada sel MCF-7, manakala 3,19-(4-bromobenzylidene)andrographolide (**SRJ08**), **SRJ09**, 3,19-(3-bromobenzylidene) andrographolide (**SRJ10**), 3,19-(3-chloro-4-fluorobenzylidene)andrographolide (**SRJ23**) and 3,19-(2-fluorobenzylidene)-andrographolide (**SRJ27**) hanya merencatkan fasa G\(_1\) pada sel MCF-7. Kesan **SRJ09** terhadap (oerangsangan hitaran regulaton cyclin) yang bergantung terhadap kinase 4 (CDK4) telah ditentukan melalui analisis Western blot. **SRJ09** merencatkan tahap CDK4 pada sel MCF-7 setelah dirawat selama 72 jam. Analisis NCI COMPARE menunjukkan mekanisme aktiviti sebatian-sebatian ini, tidak sama seperti yang ada pada dadah antikanser yang diketahui. Projeksi **SRJ03** dalam analisis ‘Self-Organising Maps’ (SOMs) mencadangkan mekanisma tindakannya berkemungkinan bersasar ke atas enzim fosfatase atau kinase. Walau bagaimanapun, andrographolide, **SRJ01**, **SRJ05**, **SRJ09** dan **SRJ13** tidak dipamerkan dalam kategori mekanisma yang diketahui.

Mekanisma kematian sel yang diaruh oleh agen baru ini dikenalpasti melalui pemerhatian mikroskop pendaflor dan ‘sitometri aliran’. Daripada kedua-dua kaedah ini, apoptosis dikenal pasti sebagai mekanisma utama kematian sel HCT-116 yang dirawat dengan **SRJ09** dan **SRJ23**.

Secara kesimpulan, sebahagian sebatian terbitan andrographolide, terutamanya **SRJ09**, **SRJ18** dan **SRJ58** menpunyai potensi sebagai komponen utama kajian antitumor untuk menemui calon klinikal yang bekesan di masa hadapan.
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I certify that an Examination Committee met on 21st October 2004 to conduct the final examination of Jada Srinivasa Rao on his Doctor of Philosophy thesis entitled “Semisynthesis of Andrographolide Derivatives and Evaluation of Their Antitumour Properties” 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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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.

JADA SRINIVASA RAO

Date: 06/12/2004
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ab-1</td>
<td>Actin</td>
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<tr>
<td>AO</td>
<td>acridine orange</td>
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<tr>
<td>AMPS</td>
<td>ammonium persulfate</td>
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<tr>
<td>AG</td>
<td>andrographolide</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>COMPARE</td>
<td>Computerised Pattern-recognition algorithm</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamino-2-phenyl indole</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic-acid</td>
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<tr>
<td>ECL</td>
<td>enzyme chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>EGTR</td>
<td>ethylene glycol-bis (β-aminoethyl ether) N, N', N''-tetraacetic acid</td>
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<tr>
<td>FACs</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GI&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% growth inhibition</td>
</tr>
</tbody>
</table>
H₂O  distilled water/sterile water
HPLC  high-pressure liquid chromatography
HRP  horseradish peroxidase
IC₅₀  50% inhibition concentration
LC₅₀  50% lethal concentration
MTT  3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NCI  National Cancer Institute
PBS  phosphate-buffered saline
PCC  Pearson correlation coefficient
PI  propidium iodide
PS  phosphatidylserine
PVDF  polyvinylidene fluoride
RNA  ribonucleic acid
RNase  ribonuclease
RPMI  Roswell Park Memorial Institute
SD  standard deviation
SDS  sodium dodecyl sulphate
SDS–PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOM  self-organising maps
TCM  traditional Chinese medicine
TEMED  N,N,N',N'-tetramethylethylenediamine
TGI  total growth inhibition
THF  tetrahydrofuran
TLC  thin layer chromatography
CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The use of plants as medicines goes back to early man. Certainly the great civilisations of the ancient Indians, Chinese, and North Africans provided written evidence of man's ingenuity in utilising plants for the treatment of a wide variety of diseases. In ancient Greece, scholars classified plants and gave descriptions of them thus aiding the identification process. It was not until the 19th century that man began to isolate the active principles of medicinal plants and one particular landmark was the discovery of quinine from *Cinchona* bark by the French scientists Caventou and Pelletier. Such discoveries led to an interest in plants from the New World and expeditions scoured the almost impenetrable jungles and forests in the quest for new medicines (reviewed by Phillipson, 2001). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today (reviewed by Phillipson, 2001).

Nature is an attractive source of new therapeutic candidate compounds and has a tremendous chemical diversity found in millions of species of plants, animals, marine organisms and microorganisms. The development of novel agents from natural sources presents obstacles that are not usually met when one deals with synthetic compounds. For instance, there may be difficulties in accessing the source of the samples, obtaining appropriate amounts of the sample, identification and isolation of the active compound in the sample, and problems in synthesising the necessary amounts of the compound of interest (Rocha *et al.*, 2001).
There are about 500,000 species of plants growing on the earth and it is estimated that at least 5000 different chemical compounds of secondary metabolites are present in a single species of plant (reviewed by Verpoorte, 1998). It is apparent that the secondary metabolites of plant origin constitute a tremendous resource for exploring useful drugs. In plants, the primary metabolites, including proteins, lipids, nucleic acids, enzymes, and coenzymes, etc., come from the metabolism of carbohydrates with the incorporation of nitrogen and mineral elements. By utilising primary metabolites and numerous infinite molecules, plants synthesise the secondary metabolites for the purpose of survival and well-being. Taxonomically related plants generally produce chemically similar secondary metabolites and, therefore, may have similar pharmacological effects. Natural products exhibiting antitumour activity continue to be the subject of extensive research aimed at the development of drugs for the treatment of different human tumours.

In the early 1950s, a research program screening for antitumour drugs of plant origin was initiated mainly by the National Cancer Institute (NCI) in the USA. Large-scale screening procedures were made available, plant materials were produced, and crude extracts were put through preliminary screening. Basic pharmacological and toxicological studies in animals ensued, and finally, a number of promising compounds were selected for chemical studies, with the ultimate goal of finding the active antitumour drugs from plants. This program represented a combined effort mobilising many biomedical research organisations in the government and in medical, pharmaceutical, and chemical institutes and industries. The achievements during the past few decades have been very rewarding (reviewed by Cragg et al., 1999).