DISCOVERY OF POTENTIAL ANTICANCER AND NEUROPROTECTIVE AGENTS FROM NORTH QUEENSLAND PLANTS

Thesis submitted by
Stephen Henry WRIGHT BSc(Hons) (Univ. of Melbourne)
in February 2005

for the degree of Doctor of Philosophy
in the Department of Chemistry
and the Department of Physiology and Pharmacology
James Cook University of North Queensland

STATEMENT OF ACCESS

I, the undersigned and author of this work, understand that James Cook University will make this thesis available for use within the University Library and, via the Australian Digital Theses network or other means, allow access to users in other approved libraries.

I understand that, as an unpublished work, a thesis has significant protection under the Copyright Act, and do not wish to place any further restriction on access to this work.

Stephen Wright February 2005

STATEMENT REGARDING THE ELECTRONIC COPY

I, the undersigned and author of this work, declare that the electronic copy provided to the James Cook University Library is an accurate copy of the printed thesis submitted, within the limits of technology available.

Stephen Wright February 2005

STATEMENT OF SOURCES

DECLARATION

I, the undersigned, declare that this thesis is my own work and has not been submitted in any form for any other degree or diploma at any university or other institution of tertiary education. Information derived from the work of others, published or unpublished, has been acknowledged in the text and a list of references is given.

Stephen Wright February 2005

STATEMENT OF THE CONTRIBUTION OF OTHERS

I, the undersigned and author of this work, acknowledge the contribution of others to this work. Financial support during this study was provided in the form of a stipend provided by the Queensland Cancer Fund (QCF George Roberts Scholarship for North Queensland), and research funding provided from two Doctoral Merit Research Grants by James Cook University. Substantial supervision was provided by Assoc. Prof. Bruce Bowden (School of Pharmacy and Molecular Sciences, JCU; primary supervisor) and Dr Anna-Marie Babey (School of Biomedical Sciences, JCU). Additional supervision was provided by Dr Vimal Kapoor (School of Medical Sciences, University of New South Wales), Dr Alan Nimmo (School of Biomedical Sciences, JCU) and Assoc. Prof. Betsy Jackes (School of Tropical Biology, JCU). Assoc. Prof. George Meehan (School of Pharmacy ad Molecular Sciences, JCU) provided additional advice on some technical and theoretical aspects. Editorial assistance in the preparation of this thesis was provided by Assoc. Prof. Bruce Bowden, Dr Anna-Marie Babey and Ms Rachael Rutkowski (School of Pharmacy and Molecular Sciences, JCU). The laboratory of Dr Vimal Kapoor in the School of Medical Sciences, UNSW, was used during this study, in addition to facilities within the School of Pharmacy and Molecular Sciences and the School of Biomedical Sciences. Dr David Newman, through the National Cancer Institute (NCI), Washington DC, provided free testing of samples for cytotoxicity in a 60 cell line panel and interpretation of results, and some samples were tested personally by Ms Sarah Wisemiller. The NCI also provided five cancer cell lines to us (Dr Anna-Marie Babey, Assoc. Prof Bruce Bowden, myself) free of charge and at their expense. Mr Rick Willis at the Australian Institute of Marine Sciences, Townsville, performed mass spectrometry (ESI-MS) on samples free of charge. Assoc. Prof. George Meehan performed optical rotation ($[\alpha]_D$) experiments on samples using facilities within the School of Chemistry, University of Sydney.

Stephen Wright February 2005

DECLARATION ON ETHICS

I, the undersigned and author of this work, declare that the research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). Research involving the use of animals followed *The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and the *Queensland Animal Care and Protection Act 2001*. The proposed research methodology for animal use received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A618_00).

Stephen Wright February 2005

ACKNOWLEDGEMENTS

I wish to thank Bruce Bowden for his supervision, advice and general support, and for guiding the metamorphosis of a botanist into a natural product chemist. I am also grateful to Anna-Marie Babey for her supervision and support, particularly in the cytotoxicity portion of my project, and Vimal Kapoor for his supervision of the enzyme inhibition aspect of my project. Thanks are extended to Betsy Jackes for botanical assistance and Alan Nimmo for his earlier supervision of my PhD and for his help in getting me established.

I am indebted to Rachael for her love and support, and for her tolerance during my thesis preparation. I am also extremely grateful to my parents, family and friends for their love and support throughout.

I wish to acknowledge and thank George Meehan for discussions and for performing optical rotations; Dr David Newman, Dr Richard Camalier, Dr Dominic Scudiero and others at the NCI for generously providing sample testing, cell lines and advice; Rick Willis for performing mass spectrometry; staff at Lavarack Barracks for permission to collect in the Mount Stuart Defence Training Area; and Prof W. Taylor (School of Chemistry, University of Sydney) for kindly providing a sample of methyl betulinate.

I am extremely grateful to the Queensland Cancer Fund for providing a three year PhD scholarship. Research funding from James Cook University (Doctoral Merit Research Grants) is also much appreciated.

I am greatly appreciative for the assistance and support of Brendan Sellors, Yunjiang Feng, Sarah Wisemiller and other past and present students of Bruce's lab. I would also like to thank Michael Ridd, Christine Hall and those who I've failed to mention due to restrictions of brevity, particular those in the School of Pharmacy and Molecular Sciences and the School of Biomedical Sciences. Lastly, for preservation of my sanity, I would like to thank the vibes of Pink Floyd and many others that hit the spot in dire times.

ABSTRACT

Plants represent a largely untapped resource for drug discovery. There are approximately 25,000 plant species in Australia alone, and 9,000 of these are found in Queensland. This study aimed to discover drug leads from North Queensland plants by screening extracts for pharmacological activity. Two pharmacological targets were selected. The first screen aimed to find novel cytotoxic compounds with potential as anticancer agents. The second screen aimed to find compounds with therapeutic potential in schizophrenia or neurological disorders that involve neuroinflammation and neurodegeneration, such as Huntington's Disease and AIDS-related dementia. Various imbalances in the relative levels of kynurenine pathway metabolites, particularly kynurenic acid, quinolinic acid and 3-hydroxykynurenine, have been implicated in these conditions. In order to discover potential drug leads that might be applied to rectify these imbalances, the extracts were screened for inhibition of three key enzymes of this pathway, namely kynurenine-3-hydroxylase, kynureninase and kynurenine aminotransferase.

Samples (365) from 125 species of plants from North Queensland were collected and extracted. After removal of tannins to prevent interference with the assays, the plant extracts were screened for cytotoxicity and for enzyme inhibition. Cytotoxicity was assessed *in vitro* using the P388D1 mouse lymphoma cell line. The enzyme inhibition assays involved the use of crude enzyme preparations from rat liver or kidney, and products were quantified by HPLC with electrochemical or fluorescence detection. Extracts with the greatest kynurenine-3-hydroxylase inhibition and selected cytotoxic extracts were subjected to bioassay-guided fractionation in order to isolate and identify the active compounds. No extracts possessed sufficient kynureninase or kynurenine aminotransferase inhibition to warrant investigation.

Three cytotoxic quinonemethide triterpenes were isolated from *Maytenus cunninghamii* (Celastraceae). Netzahualcoyoic acid (**25**, IC_{50} value = 0.12 μM, 0.11–0.13 μM 95% CI range) and Δ^{15}-celastrol (**32**, IC_{50} value not determined) are new structures, while celastrol

(**22**, IC_{50} value = 0.37 μM, 0.30–0.45 μM 95% CI range) is a known cytotoxic agent. Δ^{15}-celastrol represents a possible biosynthetic intermediate between celastrol and netzahualcoyoic acid. Further work is needed to assess the cytotoxic profile of netzahualcoyoic acid and its acid-rearranged products in the National Cancer Institute's 60 cell line panel.

The known cytostatic compounds podophyllotoxin (**36**), deoxypodophyllotoxin (**37**) and picropodophyllotoxin (**38**) were isolated from *Callitris intratropica* (Cupressaceae). This is the first reported isolation of podophyllotoxins from this species.

Bioassay-guided fractionation of kynurenine-3-hydroxylase inhibitory extracts led to the isolation of two new triterpenes, 11α,28-dihydroxylupenone (**43**) and 2α,3β-dihydroxyfriedelan-29-oic acid (**53**), from *Maytenus disperma*, as well as two new 24-oxomaytenonic acids (**58** and **60**) and four known triterpenes from *M. cunninghamii*. A biosynthetic pathway was proposed for compounds isolated from *M. cunninghamii* and related compounds. Five other species afforded a total of five additional known triterpenoids.

Triterpenes were identified as a new class of potent and selective competitive inhibitors of kynurenine-3-hydroxylase. The most active was uncaric acid (**78**, K_{ic} = 0.023 ± 0.002 μM), isolated from *Dolichandrone heterophyllum* (Bignoniaceae), and it is one of the most potent kynurenine-3-hydroxylase inhibitors that has been reported. The next most active inhibitors of this study were the 24-oxofriedelan-29-oic acids (**60**, K_{ic} = 0.061 ± 0.005 μM; **62**, K_{ic} = 0.077 ± 0.011 μM; **58**, K_{ic} = 0.12 ± 0.02 μM) and celastrol (**22**, K_{ic} = 0.14 ± 0.03 μM). Important structure-activity relationships relating to triterpene skeleton, functional groups, and ring conformations were observed. It is proposed that the triterpene inhibitors would make ideal drugs for the treatment of many neuroinflammatory and neurodegenerative disorders, and that uncaric acid, 2α-hydroxy-24-oxomaytenonic acid (**60**) and celastrol should be investigated *in vivo*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

[I]	inhibitor concentration
[S]	substrate concentration
1D	one dimensional
2D	two dimensional
3HA	3-hydroxyanthranilic acid
3HAO	3-hydroxyanthranilic acid oxidase
3HK	3-hydroxykynurenine
AA	anthranilic acid
AD	Alzheimer's Disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
aq	aqueous
br d	broad doublet
br s	broad singlet
BRI	Herbarium of Queensland, Brisbane
BSA	bovine serum albumin
C_5D_5N	deuterated pyridine
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
CH_3COOH	acetic acid
$CHCl_3$	chloroform
CI	confidence interval
CNS	central nervous system
CSF	cerebrospinal fluid
CSIRO	Commonwealth Scientific and Industrial Research Organisation
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DEPT	Distortionless Enhancement by Polarisation Transfer
dm	doublet of multiplets
DMSO	dimethylsulfoxide
EAAs	excitatory amino acids
EDTA	ethylenediaminetetraacetic acid
ESI-MS	Electrospray Ionisation Mass Spectrometry
EtOAc	ethyl acetate
EtOH	ethanol

FCS	foetal calf serum
*g*COSY	gradient Correlated Spectroscopy
*g*HMBC	gradient Heteronuclear Multiple-Bond Coherence
*g*HMQC	gradient Heteronuclear Multiple-Quantum Coherence
*g*HSQC	gradient Heteronuclear Single-Quantum Coherence
HD	Huntington's Disease
HPLC	High-Performance Liquid Chromatography
IC_{50}	with respect to cytotoxicity: defined as the concentration of compound that inhibits cell-replication by 50% relative to the control
IC_{50}	with respect to enzyme inhibition: defined as the concentration of inhibitor that reduces the formation of product by 50% relative to the control
i.d.	internal diameter
IDO	indoleamine-2,3-dioxygenase
IR	Infrared
J	coupling constant
JCT	Herbarium of James Cook University
JCU	James Cook University
KAT	kynurenine aminotransferase
K_{ic}	the specific (competitive) inhibition constant
K_{iu}	the catalytic (uncompetitive) inhibition constant
K_m	the concentration of substrate at which the rate of product formation is equal to half the maximum, or limiting, rate of product formation(V)
K_m^{app}	the apparent K_m in the presence of an inhibitor
KYN	L-kynurenine
KYNA	kynurenic acid
m	multiplet
MeCN	acetonitrile
MeOH	methanol
min	minutes
mNBA	(*m*-nitrobenzoyl)alanine (**13**)
mp	melting point
NAD	β-nicotinamide adenosine dinucleotide
NADPH	β-nicotinamide adenine dinucleotide phosphoric acid
NAL	nicotinylalanine (**11**)
NCI	National Cancer Institute, Washington DC
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
nOe	nuclear Overhauser effect

NOESY	Nuclear Overhauser Effect Spectroscopy
OD	optical density
oMBA	(*o*-methoxybenzoyl)alanine (**12**)
PCP	phencyclidine (**1**)
PD	Parkinson's Disease
PDA	photodiode-array
PLP	pyridoxal-5'-phosphate
q	quartet
QUIN	quinolinic acid
s	singlet
SAR	structure-activity relationship
sp.	species (singular)
spp.	species (plural)
SRB	sulforhodamine B
syn.	synonym
t	triplet
TCA	trichloroacetic acid
td	triplet of doublets
TDO	tryptophan-2,3-dioygenase
Tris	tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v br	very broad
V	the maximum, or limiting, rate of product formation
v	the measured rate of product formation
V^{app}	the apparent maximum rate of product formation in the presence of an inhibitor
var.	variety
Vis	Visible light