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Toxicology Investigations With The Pectenotoxin-2 Seco Acids

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy.

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Statement of originality

I hereby certify that this work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material that has previously been published or written by another person except where due reference is made in the thesis itself.

…………………………………..
Vanessa A. Burgess

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ABSTRACT

Pectenotoxins (PTXs) are a group of large cyclic polyether compounds associated with diarrhetic shellfish poisoning (DSP) as they are often found in combination with other DSPs such as okadaic acid (OA) and dinophysis toxins (DTXs) in shellfish. Although classified and regulated with the DSPs, there is debate over whether these toxins should be classified with DSP toxins. To date, ten different analogues of PTXs have been identified from shellfish and algae, and of these, the pectenotoxin-2 seco acids (PTX2-SAs) are of particular interest as they have previously been implicated in a shellfish poisoning incident in Australia, but relatively little was known of their toxicology.

One such incident occurred in December 1997, when approximately 200 people were reported with severe diarrhoetic shellfish poisoning in Northern New South Wales (NSW). Analysis of the shellfish associated with this incident revealed relatively high PTX2-SA concentrations (approx. 300μg/kg shellfish meat), with only trace amounts of pectenotoxin-2 (PTX2) and OA. Following this incident, PTX2-SAs were considered a health threat and guidelines were implemented in the absence of toxicological data, which has caused a great economic burden to shellfish industries around the globe, in particular to Australia, New Zealand and Ireland. Such regulation created in the absence of scientific data demonstrated the need to determine the toxicology of PTX2-SAs in commercial shellfish. Thus a comprehensive study on the toxicology and possible health implications of the PTX2-SAs in Australian shellfish was conducted.

PTX2-SAs were isolated in different batches from shellfish (pipis, oysters and mussels) and from algal bloom samples of *Dinophysis caudata*. Toxin extraction was conducted with several purification stages and chemical analysis was performed with high-performance liquid chromatography coupled to a tandem mass spectrometer (HPLC-MS/MS). The chemical stability of the PTX2-SAs was investigated to ensure consistency of doses between toxicology experiments. Acute dosing studies with mice were then performed and included toxicopathology investigations with light microscopy and electron microscopy, in addition to toxin distribution studies and investigation of *in vivo* lipid peroxidation. *In vitro* studies with $HepG_2$ cells included cytotoxicity assays, cell cycle investigations using flow cytometry and gene expression profiling of cells exposed to PTX2-SAs employing cDNA microarray technology.

Acute pathology studies demonstrated that the PTX2-SAs do not cause the characteristic symptoms or lesions associated with DSP toxins. No diarrhoea was observed at any dose level in mice and no deaths occurred up to the maximum dosing level of 1.6mg/kg PTX2-SA. Only one batch of PTX2-SA extract produced toxic lesions characteristic of a DSP toxin (batch 1-pilot study) but after follow up studies, it was determined that this first batch of shellfish most likely contained an additional

unidentified shellfish toxin or contaminant that co-extracted with PTX2-SAs during toxin isolation and purification procedures. This finding highlighted the importance of supporting the inclusion of the mice bioassay in procedures for shellfish toxin testing to enable detection of new toxins, and also highlighted the importance of toxin purification for toxicology studies. A significant rise in malondialdehyde excretion was observed within 24 hours of dosing mice, indicating that the PTX2-SAs may cause damage by lipid peroxidation *in vivo*.

In vitro studies showed $HepG_2$ cells to have cell cycle and gene expression changes within 24 hours of a dose of 800ng/mL PTX2-SAs. Cell cycle arrest was observed at the G_2/M checkpoint and gene expression changes included alterations in genes involved in cell cycle control, lipid metabolism and transport, lipid genesis and trace metal transport. Many genes involved in DNA repair processes were moderated at the 24 hour point, but as no apoptosis was observed up to 72 hours post dosing it is a promising indication that any DNA damage that may have been caused by the administration of PTX2-SAs was not lethal, and was able to be repaired.

In light of the information provided by toxicology investigations in this PhD, with particular reference to evidence of *in vivo* lipid peroxidation by raised levels of MDA in mouse urine, and changes in cell cycle distribution and gene expression in a cultured human cell line, it is concluded that there is potential for these toxins to induce biological changes in mammalian cells *in vivo* and *in vitro,* and hence potential for PTX2-SAs to cause health effects in humans.

During the course of this three-year study, developments in techniques for shellfish toxin identification within our laboratories have revealed that the shellfish responsible for the 1997 NSW poisoning incident contained significant concentrations of okadaic acid acyl esters that were not detected at the time of the NSW incident. Although reportedly less toxic than okadaic acid itself, the OA ester concentrations present may have been sufficient to cause the observed symptoms. It is also theorized that these esters could be hydrolyzed in the human gastro-intestinal tract to release okadaic acid. In the light of this new evidence and with no pathology lesions or symptoms of diarrhoea being observed in PTX2-SA dosing studies with mice, we now believe these OA acyl esters to be the causative agent in the 1997 NSW DSP incident and not the PTX2-SAs.

Nothing is currently known of the chronic toxicology of PTX2-SAs and thus their potential implications to public health in the long term cannot determined. The toxicology investigations in this thesis were acute studies, and it has not been established if the observed changes could be repaired or returned within normal limits without the manifestation of illness or disease occurring.

Utilizing the acute toxicology information in this thesis, a health risk assessment for consumption of PTX2-SA contaminated shellfish was performed. This risk assessment, employing numerous safety

factors essential for an incomplete data set, produced guideline values that are lower than the current recommend concentrations. To date, there has been no solid evidence that PTX2-SAs cause illness in humans – all documented incidents involving the PTX2-SAs have also included other DSP contaminants that are known to cause human illness. Pathology has not unequivocally been demonstrated in animal studies and thus, in consideration of the epidemiological evidence, PTX2-SAs cannot be considered as high a risk to public health as was previously thought. For the reasons discussed above, and weighing up risk-benefit considerations of the economic burden the current guideline values are causing to shellfish industries around the globe, it is recommended that levels of PTX2-SAs be monitored in recognition of the precautionary principle, but no longer regulated as tightly with other DSPs until such a time that toxicological or epidemiological evidence can prove that the PTX2-SAs are a DSP and are a more considerable threat to human health than has been indicated by toxicology studies in this thesis. This study has produced a substantial amount of acute toxicology data and has provided a good basis for future chronic toxicology investigations with the PTX2-SAs for regulatory purposes.

Publications and Presentations

The work of this thesis has contributed to the establishment of appropriate regulatory guidelines for levels of PTX2-SAs in commercial shellfish in Australia. Organizations that have already utilized information from this thesis include the Fisheries Research and Development Corporation, the South Australian Shellfish Quality Assurance Program and the Cawthron Institute on behalf of the Marlborough Sounds Shellfish Quality Programme, New Zealand.

Publications

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3. Burgess, V. A. & Shaw, G. (2001) Pectenotoxins - An Issue for Public Health: a review of their comparative toxicology and metabolism. Environment International. Vol. 27 (4) p. 275-283.

4. Eaglesham, G. K., Shaw G. R., Smith, M. R., Burgess, V., Moore, M. R. (2000) Human diarrhoetic shellfish poisoning incident involving dinoflagellate toxins in southeast Queensland, Australia. Toxicology Letters. TOLED5 116 (Suppl.1) p.49.

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5. Vanessa Burgess, Chi Hung Tzang, Zhong Yang, Yauo Zhang, Shen Xiaoyun, Geoff Eaglesham, Glen Shaw, and Michael R Moore. (In press). *In vitro* toxicology investigations with the PTX2-SAs.

6. Vanessa Burgess, Alan Seawright, Malcolm Jones, Geoff Eaglesham, Glen Shaw, and Michael R Moore. (In press). *In vivo* toxicology investigations with the PTX2-SAs.

Other publications during PhD

5. X. Shen, G. Shaw, W. Zhuang, W. Fong, V. Burgess, M.Yang and P. Lam. (2002) DNA microarray analysis of gene expression in mice treated with the cyanobacterial toxin cylindrospermopsin. Toxicological Sciences. Accepted for publication.

6. Seawright, A., Norris, R., Shaw, G., Burgess, V. and Moore, M. (2000) Toxicology and metabolism of the cyanobacterial toxins, cylindrospermopsin in mammals. Toxicology. Vol 148 (1), p. 75-76.

Oral presentations

- Queensland Health and Medical Annual Scientific Meeting, Brisbane, QLD. Investigation of the cause of a human diarrhoetic shellfish poisoning incident: clarification of the role of two different toxins. Dec 2002.
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Abbreviations

Standard scientific measurement abbreviations and of chemical formulas have been employed in this project. Additional less common abbreviations are listed below.

7-epi-PTX2-SA	7-epi-Pectenotoxin-2-seco acid
AA	Amino Acid
ACh	Acetylcholine
ADME	Absorption, distribution, metabolism, elimination
AFP	Alpha-fetoprotein
ANXA7	Annexin A7
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
APOL3	Apolipoprotein L, 3
ARHGAP8	Rho gtpase activating protein 8
ASNS	Asparagine synthetase
ASP	Amnesiac Shellfish Poisoning
ATP5G3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9) isoform 3
ATP5I	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e
AZA	Azaspiracids
AZP	Azaspiracid Poisoning
B/E/P	Benthic/ Epiphytic/ Phytic

BHT	Butyl-hydroxy-toluene
BTX-B1	Brevetoxin-B1
BW	Body weight
BYSL	Bystin-like
BZAP45	Basic leucine-zipper protein BZAP45
C4BPB	Complement component 4 binding protein, beta
C57BK/6J	An strain is an inbred intense black mouse
CA	California
CALM2	Calmodulin 2 (phosphorylase kinase, delta)
cAMP	Cyclicadenosine monophosphate
CANX	Calnexin
CAT	Carnithine-acetyltransferase
CCT7	Chaperonin containing TCP1, subunit 7 (eta)
CDC42	Cell division cycle 42 (GTP binding protein, 25kd)
CDK9	Cyclin-dependent kinase 9 (CDC2-related kinase)
CFP	Ciguatera fish poisoning
CNS	Central nervous system
CP	Ceruloplasmin (ferroxidase)
CTX	Ciguatoxin
CV	Coefficient of variation
CVS	Cardio-vascular system
CY (3 or 5)	Cyanine dye hybridizing to either the 3 or 5 prime
CYP7B1	Cytochrome P450, subfamily VIIB (oxysterol 7 alpha-hydroxylase), polypeptide 1
D	Desmosomes
D.	*Dinophysis* for example *D. acuminata*
DA	Domoic Acid
da	Daltons
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSP	Diarrhetic shellfish poisoning
DST	Diarrhetic shellfish toxin
DTX (-1, 2 etc)	Dinophysistoxin
EDTA	Ethylenediaminetetra acetic acid
EGTA	Ethylene glycol-bis-B-aminoethyl ether NNNN tetra acetic acid
EIF2S1	Eukaryotic translation initiation factor 2, subunit 1 (alpha, 35kd)
EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2
ELISAs	Enzyme-linked immunosorbent assay
EM	electron microscopy
ENO1	Enolase 1, (alpha)
ESI-MS	Electrospray ionisation-mass spectrometry
FBS	Foetal bovine serum
FCS	Flow cytometry systems
FDA	Food and Drugs administration
FRAP1	FK506 binding protein 12-rapamycin associated protein 1
G0/G1phase	Cell cycle check point between cell differientation and growth 1 stage
G_2/M phase	Cell cycle check point between growth 2 stage and meiosis stage
GIT	Gastrointestinal tract
GLC	Gas liquid chromatography
GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide
GTX	Gambiertoxin
GV	Guideline value
HBSS	HEPES buffered saline solution
HDAC2	Histone deacetylase 2
$HepG_2$	Human hepatoma cell line
HPLC	High performance liquid chromatography
i.p	Intraperitoneal
i.v.	Intravenous
IER3	Immediate early response 3

IF2	Translation initiation factor IF2
IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)
IOC	Intergovernmental Oceanographic commission
Kbp	Kilo-base-pairs
KIAA0101	KIAA0101 gene product
LC	Liquid chromatography
LC(50)	Lethal concentration (50%)
LC-MS	Liquid chromatography-mass spectrometry
LD	Lethal Dose
LDH	Lactatedehydrogenase
LM	Light microscopy
LOEL	Lowest observable effect level
M	Mitochondria
Mag.	Magnification
MAP-1	Modulator of apoptosis 1
MAP2	Microtubule-associated protein 2
MBD3	Methyl-cpg binding domain protein 3
MDA	Malondialdehyde
MG	Mucin and glycocalyx
MGC3207	Hypothetical protein MGC3207
MLCB	Myosin, light polypeptide, regulatory, non-sarcomeric (20kd)
MLCK	Myosin light chain kinase
MOV34-34KD	COP9 subunit 6 (MOV34 homolog, 34 kd)
MPL	Maximum permissible level
MQ	Milli Q water
MRPL19	Mitochondrial ribosomal protein L19
MS	Mass spectroscopy
MSDS	Material Safety Data Sheet
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Maitotoxin
MU	Mouse units
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5 (15kd) (NADH-coenzyme Q reductase)
NHMRC	National Health and medical research council
NMR	Nuclearmagnetic resonnance
NOEL	No observable effect level
NRU	Neutral red uptake
NSP	Neurotoxic Shellfish Poisoning
NSW	New South Wales
NZ	New Zealand
O.D.	Optical density
O1, o2, o3, or o4	Oyster extract number 1, 2 or 3 or 4
OA	Okadaic Acid
p.o	Oral administration
P1, P2 or P3	Pipis extract number 1, 2 or 3
P450s	Cytochrome P450 enzymes
PbAb	Brevetoxin antibody
PBS	Phosphate buffered saline
PbTx (-1, 2 etc)	Brevetoxins
PCR	Polymerase chain reaction
PCTK1	PCTAIRE protein kinase 1
PEG10	Paternally expressed 10
PHKG2	Phosphorylase kinase, gamma 2 (testis)
PLA2G1B	Phospholipase A2, group IB (pancreas)
PMT	Dye incorporating efficiency
PP	Protein phosphatase
PPAR	Peroxisome proliferator activated receptor
PPARg	Peroxisome proliferative activated receptor, gamma
PPE	Phosphodiesterase
PPIF	Peptidylprolyl isomerase F (cyclophilin F)

PSMC1	Proteasome (prosome, macropain) 26S subunit, atpase, 1
PSP	Paralytic Shellfish Poisoning
PTC-100	Programmable thermal controller
PTDSS1	Phosphatidylserine synthase 1
PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein
PTX (-1, 2 etc)	Pectenotoxin
PTX2-SA	Pectenotoxin-2 seco acid
QHSS	Queensland Health Scientific Services
QLD	Queensland
RDX	Radixin
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RPL31	Ribosomal protein L31
RPLP0	Ribosomal protein, large, P0
RPS24	Ribosomal protein s24
RPS27A	Ribosomal protein s27a
RPS27L	Ribosomal protein s27-like
RT	Room temperature
S Phase	DNA synthesis stage of the cell cycle
SAR, P.R. China	Special Administrative Region, Peoples Republic of China
SCLY	Putative selenocysteine lyase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SIM	Single ion monitoring
SJL/J	A strain of an inbred albino mouse
SLC25A4	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4
SSII RT	Superscript II Rnase H reverse transcriptase
STX	Saxitoxin
STXeq	Saxitoxin equivalents
TB	Terminal Bars
TCA	Trifluroacetic acid
TCEA1	Transcription elongation factor A (SII), 1
TDI	Tolerable daily
TFA	Trifluroacetic acid
TJ	Tight junction
TLC	Thin layer chromatography
TNRC11	Trinucleotide repeat containing 11 (THR-associated protein, 230 kd subunit)
TW	Terminal web area
TXN	Thioredoxin
UGT2B17	UDP glycosyltransferase 2 family, polypeptide B17
UNESCO	United National Educational scientific and Cultural Organization
USP9X	Ubiquitin specific protease 9, X chromosome (fat facets-like Drosophila)
UV	ultraviolet
V	Microvilli
VIC	Victoria
VSSC	Voltage sensitive sodium channels
YTX	Yessotoxin
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
ZNAD-fmf	Xaspase inhibitor