Preclinical *in vitro* and *in vivo* effects of purified and synthetic bioactive compounds from marine mollusc *Dicathais orbita* on colorectal cancer: Cancer prevention and toxicity study

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Abstract

Marine indole-based compounds, including precursors and isomers of the ancient purple dye, Tyrian purple, are known for their biological activity. In particular, the precursors 6-bromoisatin and tyrindoleninone from the Australian whelk, *Dicathais orbita* are compounds that have gained specific interest over the past few years for their anticancer effects in several cancer cell lines. Previous *in vivo* studies in mice by administration of *D. orbita* extract has indicated the potential for these bioactive compounds to prevent colon cancer, but with possible idiosyncratic liver toxicity. Therefore, purification of the most likely bioactive compounds (tyrindoleninone and 6-bromoisatin) from *D. orbita* could be helpful to enhance the anticancer properties and potentially reduce the toxicity associated with the crude extract. Synthetic 6-bromoisatin is commercially available, so testing the pure synthetic compound will also help confirm any activity associated with this compound. Tyrindoleninone is a compound which can be easily oxidized to other components. Therefore, stabilizing it by using antioxidants might be beneficial to increase its bioactive effects. The objective of this project was to optimize the purification of tyrindoleninone and 6-bromoisatin and examine the effects of these compounds, along with crude extract from *D. orbita*, on colorectal cancer *in vitro* and *in vivo*. The toxicity of these compounds and extracts was also assessed *in vivo* to establish the safety of these compounds in the body system.

To optimize the purification of tyrindoleninone and 6-bromoisatin, initially the compounds were separated from the extract using thin layer chromatography (TLC) using a gradient of hexane, dichloromethane and methanol. Then flash

chromatography was used to purify these compounds using the most effective solvent system from TLC. The purified compounds were analysed using liquid chromatography/mass spectrometry (LC/MS) to confirm their identity and purity. The chemical composition of crude extracts from egg masses and hypobranchial glands were also compared by LC/MS and found to contain a very similar percent composition of the main brominated compounds. In order to inhibit the degradation of tyrindoleninone, a fraction containing tyrindoleninone and tyrindolenine was exposed to oxygen overnight in the presence of two antioxidants, Vitamin A and Vitamin E, and then reanalysed by LC/MS. The synergic anti-proliferative effect of tyrindoleninone with the most effective antioxidant was then tested on HT29 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT) assay. The antioxidant experiment showed that 0.1 % Vitamin E was the most effective antioxidant for inhibiting tyrindoleninone degradation, but it failed to increase the cytotoxic effect of tyrindoleninone on HT29 cells and in fact appeared to provide some protection against the cytotoxic properties of tyrindoleninone.

In the next *in vitro* experiments, an egg mass extract was used for purification of the bioactive compounds with the optimised flash silica chromatography method. Bioassay guided fractionation was performed to identify the compounds with the greatest antiproliferative effects against colon cancer cells. The identity of the main bioactive compounds was confirmed by LC/MS, GC/MS and NMR as tyrindoleninone (>99% purity) and 6-bromoisatin (90% purity). These compounds were then tested for cytotoxic, apoptotic or necrotic effects using MTT, caspase 3/7 and membrane integrity assays respectively, on HT29 and Caco2 cells. The apoptotic effects of the bioactive compounds were confirmed by flow cytometry using Annexin-V-FITC and

PI staining. Cell cycle analysis was also performed on HT29 cells treated with the most bioactive compound. The 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay showed that semi-purified 6-bromoisatin inhibited the viability of both cell lines (IC_{50}= 100 μM). The fraction containing 6-bromoisatin activated caspase-3 and -7 enzymes in Caco2 and HT29 cells at approximately 100 μM (0.025 mg/mL) and 200 μM (0.05 mg/mL) respectively, much lower concentrations than those required to cause LDH release and necrosis (~1000 to ~2000 μM). Flow cytometry showed that semi-purified 6-bromoisatin (~200 μM) induced 77.6% apoptosis in HT29 cells. Cell cycle analysis showed the accumulation of 25.7% of HT29 cells treated with semi-purified 6-bromoisatin (~100 μM) in G2/M phase. The other compound, tyrindoleninone, was also found to inhibit the proliferation of Caco2 cells (IC_{50}= 98 μM) and HT29 (IC_{50}= 390 μM). Caspase-3 and -7 activity significantly increased only in HT29 cells treated with 195 μM (0.05 mg/mL) tyrindoleninone. LDH was released in both cell lines treated with high concentrations of tyrindoleninone.

In an *in vivo* trial, the effects of the purified tyrindoleninone and semi-purified 6-bromoisatin, along with the crude extract were tested for prevention of colorectal cancer in a two week mouse trial to determine whether these compounds can enhance the acute apoptotic response to genotoxic carcinogens (AARGC). The anti-proliferative effects of the extract and purified/semi-purified compounds were also tested by immunohistological examination using Ki-67 antibody. To evaluate any possible toxicity of the compounds, mouse general health, behavior, body weight and liver weight were assessed. Liver damage was also tested using histopathology and also biochemistry by measuring liver enzymes (ALT, AST and ALP) in the serum.

Some other biochemical and also hematological blood tests were performed to evaluate any other toxicity or side effects in blood and kidney. Semi-purified 6-bromoisatin (0.05 mg/g) was found to be the most bioactive compound in the crude extract capable of enhancing the apoptotic index in distal colon of mice. Tyrindoleninone did not increase the apoptotic index significantly. Semi-purified 6-bromoisatin did not show any toxic effect on liver, as indicated by no significant difference in the liver enzymes in comparison to the controls. In contrast, tyrindoleninone caused an increase in AST level compared to the saline control and also caused a reduction in red blood cell counts.

In my last experiment, pure synthetic 6-bromoisatin was tested for *in vitro* anticancer activity and prevention of the colorectal cancer using the same *in vivo* model. Administration of pure synthetic 6-bromoisatin to the mice, confirmed the results from the semi-purified 6-bromoisatin, with a significant increase in apoptosis at 0.05 mg/g, without any sign of toxicity in the liver or blood cells. However, a decrease in the potassium levels in the blood indicated the possibility of a diuretic effect associated with synthetic 6-bromoisatin.

This research confirmed the anticancer effects of 6-bromoisatin against two colorectal cancer cell lines *in vitro*, as well as the potential cancer preventative effects *in vivo* based on the ability to induce apoptosis in DNA damaged cells. This supports the potential development of this molluscan extract or natural 6-bromoisatin as a nutraceutical for chemoprevention of colorectal cancer. In addition, synthetic 6-bromoisatin is a promising lead for further pharmaceutical development for prevention of this disease. However, future studies in longer term animal models are required to

confirm that the early stage prevention of tumors by apoptosis in DNA damaged cells by 6-bromoistain does prevent the formation of actual tumors at the later developmental stages.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree of diploma in any university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Babak Esmaeelian

Preface

Parts of the work presented in this thesis have been submitted for publication, or are currently in preparation for submission for publication. In this thesis, Chapters 3-5 are in manuscript format and hence there is some necessary repetition in the methods and introduction sections. Chapters 2, 3 and 5 are in the format more typical for chemistry journals (e.g. *Marine Drugs*) with combined results and discussion, whereas Chapter 4 is in the format more typical for cancer journals (e.g. *Cancer Letters*), with separate results and discussion sections. All references are listed at the end of the thesis to eliminate repetition,

Manuscripts for publication:

Chapter 3. Esmaeelian, B, Benkendorff, K, Johnston, MR and Abbott, CA "Purified brominated indole derivatives from *Dicathais orbita* induce apoptosis and cell cycle arrest in colorectal cancer cell lines" This chapter has been published as a research paper in Marine Drugs on 11 October 2013.

Chapter 5. Esmaeelian, B, Benkendorff, K, Le Leu, R and Abbott, CA "Bromoisatin found in muricid mollusc extracts inhibits colon cancer cell proliferation and induces apoptosis, preventing early stage tumor formation in a colorectal cancer rodent model" This chapter has been published as a research paper in Marine Drugs on 24 December 2014.

Chapter 2. Esmaeelian, B, Abbott, CA and Benkendorff, K "Optimizing the purification of tyrindoleninone and 6-bromoisatin and testing the proliferative effect of stabilized tryindoleninone on HT29 cells" A part of this chapter (The optimization of purification) is under preparation to *BMC Complementary Medicine* but the other part (Stabilization of tyrindoleninone) will go to another Journal.

Chapter 4. Esmaeelian, B, Benkendorff, K, Le Leu, RK and Abbott, CA "Brominated indoles from a marine mollusc extract prevent early stage colon cancer formation *in vivo*" Under preparation for submission to *Cancer Letters*

Conference presentations:

Esmaeelian, B, Benkendorff, K and Abbott, CA, "Preclinical testing of purified Muricid mollusc extract in rodent models for colorectal cancer", Molluscs 2009 Conference, Brisbane, Qld, Australia, November 25- 27, 2009, Poster

Esmaeelian, B, Benkendorff, K and Abbott, CA "Anti-cancer activity of the purified bioactive compounds from Australian muricid mollusk on colorectal cancer cell lines" The 13th International Symposium on Marine Natural Products, Phuket, Thailand, October 17-22, 2010, Poster & Oral presentation

Esmaeelian, B, Abbott, CA, Benkendorff, K, Le Leu, RK and Johnston, MR "*In vitro* and preclinical testing of purified muricid mollusc extract for colorectal cancer, Postgraduate Conference, School of Biological Sciences, Flinders University, Adelaide, Australia, June 2012, Oral presentation

Esmaeelian, B, Benkendorff, K, Le Leu, RK and Abbott, CA "The preclinical effects of purified mollusk extracts in a short term mouse model for early-stage colon cancer" Eleventh Annual AACR International Conference on Frontiers in Cancer Prevention Research, Anaheim, California, October 16-19, 2012, Poster

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Abbreviations

AARGC	acute apoptotic response to genotoxic carcinogens
ACF	aberrant crypt foci
ACS	American Cancer Society
AIF	apoptosis inducing factor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AOM	azoxymethane
ANOVA	analysis of variance
APC	adenomatous polyposis coli
AST	asparate aminotransferase
CDK	cyclin dependent kinase
CE	crude extract
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
COX2	cyclo-oxygenase-2
CRC	colorectal cancer
DAPI	4',6-diamidino-2-phenylindole
DCM	dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EC	European Commission
EGFR	epidermal growth factor receptor
EM	egg mass
EMEA	European Medicines Evaluation Agency
FDA	Food and Drug Administration
FOBT	fecal occult blood test
GC/MS	gas chromatography–mass spectrometry
GSK-3	glycogen synthase kinase-3
HCAs	heterocyclic amines
Hct	hemoglobin, hematocrit
HG	hypobranchial gland

HPLC	high-performance liquid chromatography
HX	hexane
IARC	International Agency for Research on Cancer
IBD	inflammatory bowel disease
LC/MS	liquid chromatography–mass spectrometry
LDH	lactate dehydrogenase
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MMR	mismatch repair
MSI	microsatellite instability
MTT	3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NCI	National Cancer Institute
NSAIDs	non-steroidal anti-inflammatory drugs
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PI	propidium iodide
SEER	surveillance, epidemiology and end results
SRB	sulphorhodimine B reagent
Str	staurosporine
TLC	thin-layer chromatography
TNF	tumour necrosis factor
TNM	tumor node metastasis
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TYR	tyrindoleninone
VEGF	vascular endothelial growth fact