Medium Chain Fatty Acids and Wnt/β-Catenin Inhibitors as Adjunctive Colorectal Cancer Chemotherapeutic Agents

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Quote

“Faith is taking the first step even when you don’t see the whole staircase.”

Martin Luther King Jr.

In Memory

This thesis is in memory of my beloved Dad, John Leo Fauser

6th November 1912 - 24th May 1990

Dad, your brave and selfless fight with cancer gave me the inspiration and continued courage to keep searching for the answers during this PhD, so that one day no-one will suffer during chemotherapy treatment.

Dedication

This thesis is dedicated to my life-long friend, confidante and soul sister, Lee-Anne Bennett. Your unconditional love and support of me, physically, emotionally and spiritually has given me the strength and support to believe in myself, and given me the inspiration and courage to follow my heart and achieve my dreams. Words are never enough to show my gratefulness and love for you.

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Abstract

Chemotherapy remains a primary treatment for advanced stage colorectal cancer (CRC). Although more targeted chemotherapeutic agents are under development, currently prescribed cytotoxic agents target rapidly-dividing cells without discrimination between neoplastic and non-neoplastic cells, inducing debilitating side-effects with high morbidity and mortality rates. The development of less injurious chemotherapeutic agents holds promise in alleviating the negative side-effects of chemotherapy. Fatty acids (FAs) are bioactive aliphatic monocarboxylic acids categorized by the number of carbon atoms in the aliphatic chain, classified as short chain fatty acids (SCFAs) (< C8:0), medium chain fatty acids (MCFAs) (C8-14:0) and long chain fatty acids (LCFAs) (>C16:ω3-9). Exogenous applications of FAs has demonstrated anti-neoplastic properties, and FA have been suggested as adjunctive chemotherapeutic agents for the treatment of CRC. SCFAs and LCFAs have demonstrated potent anti-neoplastic properties in *in vitro* models of CRC. However, there are gaps in the literature regarding the potential anti-neoplastic properties of MCFAs in CRC. Endogenous adjunctive CRC therapies manipulate signalling pathways related to the instigation and progression of CRC, and have only recently been incorporated into standard chemotherapeutic protocols. The Wnt/β-catenin signalling pathway is dysregulated in the majority of CRC cases leading to up-regulation of cell proliferation, crypt expansion and mutated intestinal stem cells (ISC). Inhibition of this pathway has demonstrated a down-regulation of cell proliferation. However, the effect on the process of crypt fission and ISC expression is unknown. *In vitro* (cell culture) technologies are underutilised in investigations of the biological effect and mechanisms of actions of pharmacological agents, bioactive agents and anti-neoplastic agents, when related to intestinal function. There is a paucity of intestinal non-transformed cell lines due to difficulties in the derivation and culture of these

cells. This thesis investigates the effects of a MCFA on a CRC cell line (Caco-2) and a non-neoplastic intestinal cell line (IEC-6), and the *in vivo* endogenous application of Dickkopf-1 (Dkk-1), a Wnt signalling pathway inhibitor. Furthermore, this thesis describes the derivation of a short term porcine small intestinal cell line.

Initially in **Chapter 2**, Caco-2 cells were treated with increasing concentrations of the MCFA, Lauric acid (LA) (C12:0), with cell death compared to the established anti-neoplastic SCFA, butyrate (C4:0) to induce cell death or cytotoxicity, using a CRC *in vitro* model. This study demonstrated the novel finding that LA exerts cytotoxic properties at doses of 0.5mM and 1mM, having induced a significant reduction in cell viability in Caco-2 cells, compared to butyrate and negative controls, as ascertained by trypan blue exclusion assay. This data is supported by published cytotoxic studies in lymphoblastic cells. It has been proposed that anti-neoplastic properties of FAs are carbon atom chain length dependent. On this basis, preferential cytotoxic properties of MCFAs of differing carbon atom chain lengths were investigated. LA exerted preferential cytotoxic properties compared to C10:0 and C14:0 MCFAs. Few studies simultaneously investigate the potential negative side-effects of FAs on non-neoplastic intestinal cell lines. In **Chapter 2**, the murine small intestinal cell line, IEC-6 was treated with cytotoxic doses of LA. LA induced significant cell death compared to butyrate and negative control cells. This data supported further investigation into the mechanisms underpinning cell death induced by LA in Caco-2 and IEC-6 cell lines.

Using flow cytometric analysis compared to butyrate, **Chapter 3** demonstrated that cytotoxic doses of LA induced a significant increase in the percentage of cells undergoing apoptosis as opposed to necrosis in Caco-2 cells. The new discovery that LA induced apoptosis in Caco-2 cells supported further exploration into the anti-neoplastic mechanisms of LA. Few studies

have explored the effects of intracellular redox modulation of intestinal cell homeostasis, thus this study investigated the effect of LA on reduced glutathione (GSH) levels as measured by enzymatic analysis, ROS generation and modification to cell cycle phases were measured using flow cytometric analysis. It was determined that LA reduced the availability of GSH, thereby demonstrating that LA modulated the redox system of the Caco-2 cell line. A significant increase in levels of ROS was detected in LA-treated Caco-2 cells compared to butyrate, indicating that LA induced a higher oxidative state than butyrate. Therefore, the reduction in all phases of the cell cycle in LA treated Caco-2 cells may not have been the key contributor to the induction of apoptosis. Butyrate induced significant apoptosis in Caco-2 cells compared to PBS-treated controls, reduced GSH levels equivalent to LA, decreased cells in G0/G1 phases and generated less ROS than LA. These different effects are proposed to be due to the different carbon atom chain lengths of butyrate and LA. LA at cytotoxic doses significantly reduced cell viability in IEC-6 cells, associated with a reduction in GSH, generation of ROS and modification to all cell cycle phases. Butyrate on the other hand did not decrease IEC-6 viability, reduce GSH, modify phases of the cell cycle or generate ROS, indicating that this SCFA did not influence the redox system of this cell line. This may have been related to the genetic differences in non-transformed (p53 and Wnt positive wild type) and transformed cell lines (p53 and Wnt signalling mutant).

Mutations in Wnt/β-catenin are present in the majority of CRC cases, resulting in over-expression of the downstream molecule, β-catenin, inducing uncontrolled cell proliferation leading to up-regulation of crypt fission, a precursor and promoter of CRC polyps, thereby generating a greater number of crypts which produce mutated intestinal stem cells to populate CRC tumours. Dickkopf (Dkk-1) is a pan negative regulator of the Wnt/β-catenin signalling system. In **Chapter 4** neonatal rats (day 11-15) were treated with increasing doses of Dkk-1

(30ng and 100ng). A microdissection technique in the small intestine demonstrated that both doses of Dkk-1 reduced villus and crypt areas significantly. Therefore, it was concluded that Dkk-1 reduced crypt fission but not crypt hyperplasia in neonatal rats. The Wnt controlled intestinal stem cell (ISC) marker *Lgr5* mRNA was significantly reduced in Dkk-1 treated animals in both the small and large intestine. Expression of other ISC markers, *DCAMKL-1*, *Bmi1* and *OLFM4* mRNA remained unchanged in the small intestine. In the large intestine the ISC marker *DCAMKL*-1 mRNA expression remained unchanged. *Bmi1* and *OLFM4* mRNA expression was significantly reduced. Protein expression of the Wnt downstream effector molecule, β-catenin, as measured by immunohistochemistry, was significantly reduced in the small intestine of Dkk-1 treated animals.

Rapid and easily reproducible cell culture models are essential for expanding studies of intestinal function and investigations into pharmacological and nutraceutical agents, concomitantly reducing the use of research animals. In **Chapter 5** a hybrid of intestinal primary cell isolation methods was used to derive a short-term porcine small intestinal cell line. Cells were isolated from the jejunum and ileum of a stillborn large white piglet, and, for the first time, maintained in culture for a 5 week period before reaching senescence. This demonstrates that a non-transformed epithelial cell line can be generated using basic cell culture practices, leading to the increased capacity to evaluated bioactive molecules on normal intestinal epithelial cells.

In conclusion, this thesis has provided new evidence into the anti-neoplastic properties of MCFAs in a CRC model. The MCFA, LA modulated the intracellular redox system, inducing apoptosis in Caco-2 cells, with short carbon atom chain FAs inducing a lesser degree of cell death. This finding is important as it demonstrates that carbon atom chain length influences

the rate of apoptosis and possibly the mechanisms underpinning this cytotoxicity. Thus, FAs hold promise to augment adjunctive chemotherapeutic agents. It is essential that FAs of all carbon atom chain lengths are evaluated both *in vitro* and *in vivo* for anti-neoplastic potential. Another key finding of this thesis was that crypt fission is negatively regulated by inhibition of the Wnt signalling system, critical in reducing the number of neoplastic adenomas and mutated ISC populations. This finding has potential for the use of negative regulators of the Wnt/β-catenin signalling system as possible adjunctive CRC chemotherapeutic agents. Finally, a new method to derive an intestinal cell line was developed. This simple method opens up wider scientific opportunities to evaluate the effects of bioactive compounds on species-specific non-transformed intestinal cells.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Publications and Presentations Arising from Ph.D Candidature

Publications:

Fauser J.K, Rino P. Donato, Joshua A. Woenig, Simon J. Proctor, Andrew P. Trotta, Phulwinder K. Grover, Gordon S. Howarth, Irmeli A. Penttila, and Adrian G. Cummins. Wnt blockade with dickkopf reduces intestinal crypt fission and intestinal growth in infant rats. *Journal of Paediatric Gastroenterology and Nutrition, 2012 Jul 55(1) 26-31.*

Fauser JK, LD Prisciandaro, Cummins AG and Howarth GS. Fatty Acids as adjunctive potential colorectal chemotherapeutic agents. *Cancer Biology and Therapy. 2011. 11(8):724-31.*

Whitford EJ, AG Cummins, RN Butler, **JK Fauser**, R Yazbeck, A Lawrence, KY Cheah, TH Wright, LD Prisciandaro and GS Howarth. The new probiotic, Streptococcus thermophilus TH-4, reduces crypt fission in a rat model of 5-Fluorouracil induced mucositis. *Cancer Biology and Therapy, 2009. 8(6):505-11.*

Fauser JK, Matthews, GM, Cummins AG, and Howarth GS. Modulation of intracellular redox state and induction of apoptosis by a Medium chain Length Fatty acid in a Colorectal and non-transformed intestinal cell line. *Manuscript prepared for submission to the Journal of Chemotherapy.*

See Appendix for bound copies of published articles

Abstracts and Presentations:

Fauser JK, Butler RN, Cummins AG and Howarth GS. Nutritional Supplementation for Improvement of Intestinal function and Porcine Health Subprogram 2b: *Oral presentation at the Pork CRC Annual meeting, Melbourne, November 2006*

Fauser JK, Howarth GS, Butler RN and Cummins AG. Inhibition of Wnt Signalling During Postnatal Growth of the Small Intestine. *Oral presentation at Australian Society for Medical Research, State meeting, Adelaide, June 2007*

Fauser JK, Butler RN, Cummins AG, Matthews GM and Howarth GS. Medium-Chain Fatty Acid Induction of Cell Death in the Caco-2 and IEC-6 Cell Lines. *Poster presentation at Australian Society for Medical Research, State meeting, Adelaide, June 2007*

Fauser JK, Butler RN, Cummins AG, Matthews GM and Howarth GS. Medium-chain fatty Acid modulation of cell viability in the Caco-2 and IEC-6 cell lines. *Oral presentation at Australian Gastrological Week, Perth October 2007.*

Fauser JK, Butler RN, Cummins AG, Matthews GM and Howarth GS. Medium-Chain Fatty Acid Induction of Apoptosis in Colon Cancer. *Poster presentation, Lorne Cancer Conference, Melbourne, February 2007*

Fauser JK, Howarth GS, Butler RN and Cummins AG. Wnt Blockade During Postnatal Growth of the Small Intestine Preferentially Reduces Crypt Fission and not Crypt Hyperplasia. *Poster presentation Digestive Diseases Week, California, USA, May 2008. Abstract published in Journal of Gastroenterology and Hepatology; 2008*

Jeyaraman KV, Donato RP, **Fauser JK**, and Cummins AG. Investigation of Short-term blockade of Notch Signalling on Postnatal Growth of the Small Intestine. *Poster presentation Australian Society for Medical Research, National meeting, Brisbane November 2008*

Prisciandaro LD, **Fauser JK,** Butler RN, Cummins AG and Howarth GS. Soluble products secreted from the newly identified probiotic Lactobacillus fermentum BR11 improves viability of rat intestinal cells. *Oral presentation, Nutritional Society Annual Conference, Adelaide, December 2008*

Fauser JK, Butler RN, Cummins AG, and Howarth GS. Modulation of the intracellular redox state induces apoptosis in a Colorectal cancer cell line. *Oral presentation at Australian Society for Medical Research, State meeting, Adelaide, June 2009*

Fauser JK, Butler RN, Cummins AG, and Howarth GS. Fatty Acids: Novel Bioactive Anti-Cancer Therapeutics? *Oral presentation World Congress on Oils and Fats & 28th ISF Congress, Sydney, September 2009*

Donato R, **Fauser JK,** Penttila IA, Roberts-Thomson IC, Cummins AG. Wnt ligand expression and regulation of stem cells during postnatal growth of the small intestine in rats. *Poster presentation Digestive Diseases Week, Chicago, USA, May 20011. Abstract published in Journal of Gastroenterology and Hepatology*

Abbreviations

α	Alpha
β	Beta
Δ	Delta
μg	Microgram
μM	Micromolar
μl	Microliter
nM	Nanomolar
ω	Omega
Ψ	Psi
Ψm	Mitochondrial membrane potential
5-FU	5-flurouracil
%	Percentage
±	Plus or minus
AA	Arachidonic acid
ANOVA	Analysis of variance
APC	Adenomatous Polyposis Coli
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
C	Carbon
ºC	Degree Celsius
CA	Capric acid
Ca^{2+}	Calcium
CaCl	Calcium chloride
CDMEM	Complete Dulbecco's Modified Eagle Media

CLA	Conjugated Linoleic Acid
CO_2	Carbon Dioxide
COOH	Carboxylic acid
CRC	Colorectal cancer
DAPI	4′,6-diamindino-2-phenylindole
DCF-DA	2′,7′-Dichlorofluorescin diacetate
Dkk-1	Dickkopf
DISC	Death-inducing signalling complex
DHA	Docosahexanoic acid
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic acid
DSH	Dishevelled protein
E_0	Standard potential for redox couple at a defined pH
E_h	Standard Redox Potential
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immuosorbent Assay
EPA	Eicosapentanoic acid
EtOH	Ethanol
F	Faraday’s constant
FA	Fatty acid
FACS	Fluorescence-activated cell sorting
FAP	Familial cancer
FAF-BSA	Fatty acid free serum albumin
FCM	Flow cytometric analysis
FITC	Fluorescein isothiocyonate
FZ	Frizzled receptors

g	Relative centrifuge force
G6PDH	Glucose-6-phosphate dehydrogenase
GM	Growth Media
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSSG	Oxidised glutathione
GTP	Guanosine triphosphate
HDACI	Histone deacetylase inhibitors
HI-FCS	Inactivated fetal calf serum
H	Electron
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
Hr(s)	Hour(s)
IU	International units
IP	Intraperitoneal injection
ISC(s)	Intestinal Stem Cell(s)
KCl	Potassium chloride
Kg	Kilo grams
LA	Lauric acid
LCFA	Long Chain Fatty Acid
Lef	Lymphocyte factor
Lgr5	Leucine-rich repeat containing G-protein-coupled receptor 5
LI	Large Intestine
LOA	Linoleic acid
MA	Myristic acid
Mab	Monoclonal antibody

MCFA	Medium Chain Fatty Acid
Min(s)	Minute(s)
ml	Milli Liter
mRNA	messenger RNA
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
Msi-1	Musashi-1
n	Number of electrons
$NaCl_2$	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NaH_2PO_4	Sodium hydrogen carbonate
Na_2PO_4	Sodium dihydrogen phosphate
NF-κβ	Necrosis factor kappa-light-chain
ng	Nanogram
n.s	Not sampled
N/S	Not significant
$^*O^{2-}$	Superoxide anion
OA	Oleic acid
OD	Optical density
$^*OH^-$,	Hydroxyl radical
OLFM4	Olfactomedin 4
OPP	Oxidative pentose pathway
PI3K	Phosphoinositide-3-kinase
PARP	Poly ADP-ribose polymerase

PBS	Phosphate buffered saline
PI	Propidium iodide
PMA	Palmitic acid
PPAR	Peroxisome proliferation-activated
PS	Phosphatidylserine
R	Gas constant
RNA	Ribonucleic acid
ROS	Reactive oxygen species
R_T	Trans-epithelial resistance
RT	Room temperature
SCFA	Short Chain Fatty Acid
SI	Small Intestine
siRNA	Small interfering RNA
T	Absolute temperature
Tcf	T cell factor
TCF(s)	Tissue culture flasks
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TCP(s)	Tissue culture plate(s)
Trx	Thioredoxin
Trx(SH)	Thioredoxin (reduced)
UC	Ulcerative colitis
VEGF	Vascular epithelial growth factor
Wt	Wild type