The therapeutic applications of medium-chained saturated fatty acids in the treatment and prevention of intestinal protozoal infections

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Dedicated to everyone who helped make me who I am today,

for I am a day closer to fulfilling my dreams

because of you!
Statement of Originality

The material presented in this thesis has not been previously submitted for a degree or diploma in any university, and to the best of my knowledge, contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

Signature:

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Paran Rayan

October 2009
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Abstract

*Giardia duodenalis* is a protozoal, intestinal parasite that is a common aetiological agent of infectious diarrhoea in humans worldwide. Chemotherapeutic intervention presently offers a limited range of drugs and these are usually only employed after clinical diagnosis. Moreover, these drugs are ineffective against the infectious cysts, can produce unpleasant side effects, and are expensive with limited availability in developing countries. Frequent reports of drug toxicity, treatment failure and parasite drug resistance have, in some instances, also resulted in the increasing reluctance to over-prescribe synthetic anti-microbials. Alternatively, there is now mounting evidence to suggest that some of the naturally derived, medium-chain, saturated fatty acids (MCSFAs) possess anti-microbial and anti-parasitic properties.

I have therefore examined the effects of four different fatty acids on *G. duodenalis* trophozoites in vitro. Cytotoxicity was determined using fluorescence, scanning and transmission electron microscopic techniques and standard cytotoxicity assays. My studies have confirmed that the MCSFA, dodecanoic acid (C:12; common name lauric acid; DA), is anti-giardial with an LD$_{50}$ concentration comparable to that of metronidazole, the drug of choice in the treatment of giardiasis. DA appears to induce trophozoite death by accumulating within the parasite cytoplasm resulting in rupture of the cell membrane.

*In vivo* trials in mice using DA and coconut oil (which consists of 45-55% DA) as dietary supplements have also appeared promising against *G. duodenalis* infection, however the water soluble derivative, monolaurin (C:15; dodecanoic acid, 2,3-dihydroxypropyl ester) did not. These studies have opened fresh avenues for development of natural drug therapy in which food supplementation may augment, or even replace, some of the standard chemotherapeutic agents presently employed in the treatment of giardiasis and possibly other infectious intestinal diseases.

In order to develop an alternate model to further our research goals, 3-D tissues grown in vitro were used to explore disease pathogenesis, which could possibly replace expensive experimental animal models. Tissue engineering is a relatively modern and expanding field of medical research working to
develop appropriate models and technologies to promote the regeneration of human tissues. This includes the restoration of tissue and organ function via creating biological substitutes in place of harvested tissues, artificial implants and prostheses. Full clinical regeneration of a tissue encompasses the source of suitable cell types, encouraging these cells to grow into an appropriate, fully functioning, three-dimensional (3-D) tissue, and incorporating this tissue successfully into the recipient. Likewise, three dimensional tissues employed in medical research would reduce the need for several animal experimentation protocols, particularly in the fields of tropical medicine with emphasis on parasitology.

The extracellular matrix (ECM), consisting of the organised network of extracellular materials that surrounds living cells, is an important component of all tissues and organs. My research has attempted to grow an epithelial cell line – CaCO2 on the 3-D scaffold. The 3D tissue constitute was then inoculated with Giardia duodenalis trophozoites. The 3-D tissue constitute and trophozoites scaffold seeded with mammalian cell lines and trophozoites were grown for set periods of time and then observed microscopically using light and scanning electron microscopies. *In vitro* demonstration of clinical pathology such as microvillus blunting was observed even after only 1 hour post inoculation. This 3-D model explored the applicability of an *in vitro* model for host-parasite co-culture and disease pathogenesis. The practical significance of these studies paves the way for future drug trials using these *in vitro* alternatives.

A pilot ‘preliminary’ study was undertaken where faecal samples from 195 school children (rural = 95; male = 39; female = 56) (urban = 100; male = 60; female = 40) of five age groups ranging from 5 to 11 years in two different socio-economic zones (rural and urban) were screened for specific intestinal parasites. Percentage prevalences of parasitic species found in faecal wet mounts and concentrates in rural children were: *Entamoeba coli* (25.3%), *Giardia lamblia* (17.9%), *Blastocystis hominis* (14.7%), *E. histolytica* (4.2%), *Iodamoeba butschlii* (1.1%), *Hymenolepis nana* (1.1%), *Ascaris lumbricoides* (1.1%), *Ancylostoma duodenale* (0%) and *Trichuris trichiura* (0%). The percentage incidences in urban children were: *Entamoeba coli* (26%), *A. lumbricoides* (21%), *B. hominis* (18%), *G. duodenalis* (14%), *T. trichiura* (8%), *I. butschlii* (4%), *A. duodenale* (1%), *E. histolytica* (0%), and *H. nana*.
These data indicate that there were greater incidences of *I. butschlii*, *B. hominis*, *A. lumbricoides* and *T. trichiura* infections in urban children compared to rural children, but that both populations had high (over 25%) incidence rates of *Entamoeba coli*. Nematode infestations, in particular *A. lumbricoides* and *T. trichiura*, were found in twice the number of urban as rural children. Such findings may be related to dietary differences, living conditions and the greater use of natural anti-helminthic medicinal plants in rural communities. These results are important for both epidemiological data collection and correlating dietary differences to intestinal parasitic diseases.

Dietary supplements of coconut oil, gingellee oil and Monolaurin were offered to 1128 school children from 3 States (Tamil Nadu, Kerala and Maharashtra) in India and then screened for intestinal parasites. The participant age groups ranged from 2-16 years and their sex ratio was 534 male and 594 female. These studies compared the prevalence rates of intestinal helminthic infestations and pathogenic protozoal infections, (in particular *G. duodenalis*), in populations that were offered supplemements of coconut oil and Monolaurin. In addition, these studies have also determined that the children from rural communities appeared to have reduced prevalences of intestinal parasites when compared to their urban counterparts. In summary, certain populations within both rural and urban communities clearly showed that supplemements of coconut oil or Monolaurin reduced helminthic and protozoal intestinal infections. These results are intriguing and further investigations are warranted in order to determine the exact factors behind these results. Various factors that could possibly affect the parasitic burdens, infection rates, and susceptibility were investigated and documented. It is anticipated that the novel compound (dodecanoic acid) under investigation in this doctoral research project may have some future application in parasitic infection treatment strategies. To date, these studies are the only human trials that have attempted to examine the antiparasitic effects of products containing the MCSFA, dodecanoic acid and Monolaurin.
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2.28 TEM micrographs of a negative control trophozoite after treatment with 1%DMSO for 15 minutes

2.29 TEM micrographs of a negative control trophozoite after treatment with 1%DMSO for 15 minutes

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3.2 Scanning electron micrographs of modified spongin scaffold with CaCo2 cells grown for 14 days

3.3 Scanning electron micrographs of modified spongin scaffold

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3.4 Scanning electron micrographs of spongin scaffold with CaCo2 cells grown 14 days and inoculated with *Giardia duodenalis* trophozoites for 3 hours

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6.31 Images of the area within the suburbs of Porur, the local fish market

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6.41 The district of Ernakulum in the state of Kerala, India;

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List of Abbreviations

- AIDS Acquired Immune Deficiency Syndrome
- ASTM American Society for Testing and Materials
- BODIPY 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene
- °C Degree Centigrade
- DA Dodecanoic acid
- DMSO Dimethyl sulphoxide
- CoA Coenzyme A
- DNA Deoxyribonucleic acid
- ECM Extracellular Matrix
- EILISA Enzyme-linked immunosorbent assay
- EPA Environmental Protection Agency
- ER Endoplasmic reticulum
- ESV Encystation-specific vesicles
- FA Fatty acid
- GalNAc N-acetylgalactosamine
- GlcNAc N-acetylglucosamine
- GPL Glycerophospholipid
- g or gm Grams
- g Acceleration due to gravity
- IC_{50} Inhibitory concentration
- LD_{50} Lethal dose
- LDL Low density lipoprotein
- LPL Lysophospholipids
- MCSFA Medium-chain saturated fatty acid
- MG Monoglycerides
- mg Milligrams
- mls Millilitres
- MUFA Monounsaturated fatty acid
- PC Phosphatidylcholine
- PCR Polymerase Chain Reaction
- pi Post inoculation
- PLA_{1} Phospholipase A_{1}
- PLA_{2} Phospholipase A_{2}
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PtdyGly</td>
<td>Phosphatidylglycerol</td>
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<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>T₁/₂</td>
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<td>TYI-S-33</td>
<td>Tryptone yeast iron-S-33</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<tr>
<td>VSP</td>
<td>Variant surface protein</td>
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