CHAPTER 2
Methodology

2.1. The Azoxymethane-Rat model of Colorectal Carcinogenesis

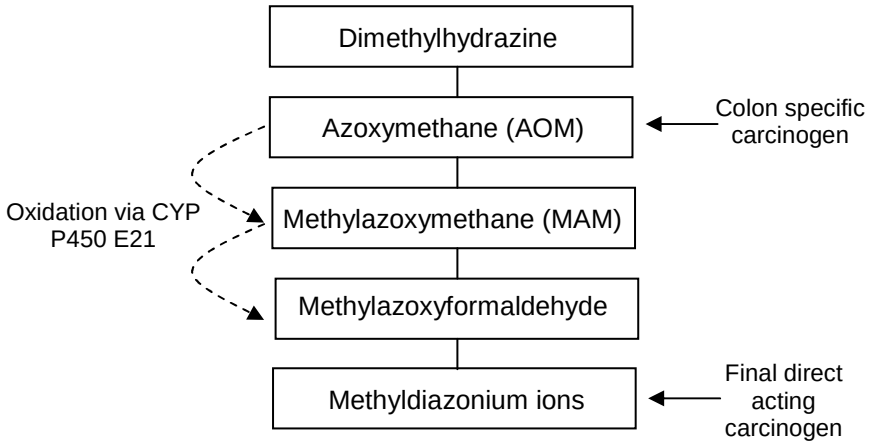
Due to the experimental nature of this project and the nature of the endpoints being tested it was not feasible to do this work using human subjects. Additionally, the use of an *in vitro* model was thought to be impractical because of the significant dietary intervention aspect of this study. Therefore, it was decided that an *in vivo* animal model would be used for all experiments.

Rodents do not spontaneously develop colorectal cancer. Therefore, a variety of models have been established to observe carcinogenesis development in the colon using chemical carcinogens. Perhaps the most popular model used in the literature is the azoxymethane (AOM) rat model. This *in vivo* model has been extensively used by researchers to induce tumourigenesis in the colon and study the chemopreventative effects of dietary agents on CRC [136, 154-156].

Derived from Dimethylhydrazine (DMH), AOM is a colon specific alkylating agent. Catalysed by the cytochrome P450 2E1, otherwise known as chloroxanzone 6-hyroxylation, AOM is broken down to methylazoxymethanol (MAM) by the hydroxylation of methyl groups. It is possible that other P450 isozymes may also be involved in this process [157]. MAM can also be oxidised by CYP2E1 in the liver and other extrahepatic organs to form methylazoxyformaldehdye. This oxidative step goes on to yield methyldiazonium ions [158].

Ultimately, these methyldiazonium ions act as the final carcinogen and go on to methylate cellular DNA. This process is outlined in figure 5. These acute methylation adducts then progress on to cause base mispairs, fixed mutations and with time, colorectal carcinogenesis.

Figure 5: The metabolism of AOM



The development of AOM induced colonic tumours in rat distal colon follows a very similar pathway and has a comparable histological appearance to spontaneous carcinogenesis development in the human colon [159]. The initiation and promotional stages of the disease are clearly distinguishable from one another in this model. The initial acute adduct formation is followed by the development of aberrant crypt foci (ACF) which are early smaller preneoplastic lesions that can then give rise to the larger adenocarcinomas [160]. As seen in human colorectal cancers, AOM-induced ACF and adenocarcinomas also display a high incidence of K-ras and beta-catenin mutations while also showing microsatellite instability [161-163]. However, the p53 gene and APC genes which are crucial in the development of the hereditary disease FAP are less likely to be mutated in this model [40].

These similarities make this *in vivo* rat model suitable to test the effects of either dietary agents or drug interventions on the initiation and development of colorectal cancer. With the ultimate goal being to eventually translate the appropriate interventions into human clinical trials. After reviewing the literature, Corpet and Pierre [164] noted that the effects of agents tested in rat models went on to give consistent results across both animal and clinical trials validating the fact that using a rodent model such as the AOM rat model is of great use in providing direction to the selection of chemopreventative strategies to human CRC.

2.2. Animals

Male Sprague-Dawley rats were used for all experiments as they are reasonably easy to work with and have a similar colonic histology and carcinogenesis development to that of humans. Rats were 4-5 weeks old and weighed approximately 150g at the start of each experiment. They were obtained from the Animal Resource Centre (Canning Vale, Western Australia).

All animals were housed at the Flinders University animal facility and maintained in a temperature and humidity controlled environment with a 12h light/dark cycle. Rats were caged in groups of 4 on raised grid floors to minimise the consumption of bedding and coprophagy and were fed and watered *ad libitum*.

All handling and experimental work with the animals was done by trained personnel and the euthanasia of all rats was carried out by CO_2 asphyxiation.

2.3. Ethics

Ethics approval was gained from the Flinders Medical Centre and the Flinders University of South Australia for all experiments completed throughout the duration of this project (approval number 590/04). While still allowing for statistical power in each experiment, the number of rats for each study was kept at a minimum and all procedures involving animals were carried out with minimal distress on the animal.

All rats used have been monitored carefully and well looked after with all guidelines and principles in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes being stringently followed.

2.4. Chemicals

Azoxymethane (A-2853) and Methoxyamine Hydrochloride (226904) were purchased from Sigma Chemicals (St Louis, MO).

2.5. Diets

All diets were made on site and were a modified version of the AIN-76 diet [165] with a protein: carbohydrate: fat content of 20:50:20 respectively. All diets were freshly made, stored at -20°C and were fed daily to the animals to avoid oxidation of diets.

The carbohydrate component of the base diet constituted a mixture of 60% cornstarch and 40% sucrose. The protein component was added to the diet in the form of casein and fibre was given as α-cellulose. Though type and ratios of lipid used varied depending the experimental requirements, lipid content remained at a consistent level of 20% throughout all experiments. All minerals and vitamins were given in compliance with the AIN -76 guidelines, including supplementations of both choline and methionine (see appendix A for details of mineral and vitamin list).

Table 1: Composition of Control diet

Diet Ingredients	g/100g total diet
Cornstarch	30
Sucrose	20
Casein	20
α - cellulose	5
Sunflower seed oil	20
Minerals	3.5
Vitamins	1
Choline	0.3
Methionine	0.2
Total	100

Sunflower seed oil high in linoleic acid (18:2n6) (Goodman Fielder Pty. Ltd., West Footscray, Victoria, Australia) was used as the control lipid, while HIDHA® 25N tuna oil was obtained from the Clover Corporation Ltd. (Altona North, Victoria, Australia) and contained a total of 35% omega-3 fatty acids, with a DHA/EPA ratio of 26.5:5.3. All oils used in the production of the microencapsulate material were identical to the free oils added to diets. Menhaden oil (omega3 18:12, HO307) used in the ACF study was obtained from

Lysi H.F (Reykjavik, Iceland) and had a DHA/EPA ratio of 13.0:19.1. To avoid the oxidation of fish oils, all containers were flushed with nitrogen after use and were stored at 4°C.

Experimental diets were manipulated with regard to their lipid type, dose and form (free or encapsulated). For detailed composition of experimental fish oil diets please refer to the acute study design in 5.1.3 and the ACF study design in 7.1.3.

2.5.1. Encapsulation of fish oil

The design and production of the microencapsulated product was carried out by Food Sciences Australia (FSA) in Werribee, Victoria using registered MicroMAX technology. This novel microencapsulation technique was designed to not only achieve a greater stability of bioactives in food products but also to deliver bioactives to specific sites in the GI tract after consumption. In this case, it was decided that the bioactive being tested would be tuna oil due to the positive impact omega-3 PUFAs have on one's health and the various publications supporting its links to a reduction of CRC risk as detailed in section1.4.4. – 1.4.6.

The encapsulated oil product is created by emulsifying both the oil and the encapsulant materials together. After a stable oil emulsion has been formed between these two components, the product is then put through a high powered spray drying process which results in the final powder-like microencapsulated oil product.

A variety of materials can be used to form the outer capsule depending on what properties are required of the final product. In this case, a specific protein, carbohydrate mix was used which gave the most promising results that suggested inhibition against the early uptake of oil in the stomach and upper gastrointestinal tract and the high delivery of oil directly to the colon instead (unpublished data).

2.6. Additional Assays

The counting of both apoptotic and proliferating nuclei were both done on formalin fixed paraffin embedded tissue. Formalin fixation was for a period of 18h in 10% buffered formalin at which point tissues were placed in 70% ethanol. Tissues were then subjected to a series of alcohol and xylene gradients overnight in a Shandon Duplex processor (See appendix B for processing details). Processed tissue samples were then embedded into hot paraffin wax using a Tissue Tek II embedding instrument. Using a Leica microtome, distal colonic tissue was cut at a thickness of 4µm so that the colonic crypts were sliced axially. These sections were then heat fixed to a poly-lysine coated slide by placing them in the oven for 30 mins.

All counting was done by eye and was carried out using an Olympus BX41 light microscope under 40x magnification. 20 crypts were counted for each rat. Each crypt counted was chosen on the basis that it had to be an intact, whole crypt with a clear, single row of epithelial cells from base to the surface of the crypt without interference from any histological artefact or otherwise. Crypts were halved and counts began at the bottom of the crypt and finished at the first vertical surface cell. All counts were done blindly.

The AARGC or the proliferation rate of cells were expressed either as the number per crypt unit structure (number of positive cells per crypt) or as a % index (number of positive cells per crypt, divided by the number of cells in a crypt).

2.6.1. Apoptosis (Haematoxylin staining)

Slides with tissue sections affixed, were placed in Histoclear II (National diagnostics, HS_20_2) for 10 mins and then dehydrated in a graded series of ethanol from 100%, 95%, 70% and 50% for 2 mins each. Slides were then rinsed in dH_2O, placed in haematoxylin for 3 minutes and rinsed again. All slides were then briefly dunked in 1% acid ethanol, rinsed in dH_2O, dunked in 1% ammonia

water and rinsed in dH_2O again. Slides were then rehydrated back through the graded ethanol series, soaked in histoclear and cover slipped.

Apoptotic cells were identified as having a condensed, dark nucleus that may include some form of blebbing or irregularity. A clear halo around this characteristic nucleus was also an indication of an apoptotic cell [76].

2.6.2. Apoptosis (TUNEL assay)

The TUNEL assay was carried out using the TdT-FragEL DNA fragmentation kit (Calbiochem, QIA33). The exposed 3`-OH ends of DNA in apoptotic cells were targeted in this assay and were positively stained brown in colour in contrast to the negative green cellular nuclei.

Slides were deparaffinised in histoclear for 10 mins and then dehydrated in a graded series of ethanol from 100% – 50%. Following a PBS rinse each tissue specimen was circled with a PAP pen and incubated with 20µg/ml proteinase K for 20 mins. Slides were then rinsed with PBS and covered with 3% H_2O_2 in methanol to minimise background staining via endogenous peroxidase activity. After a further PBS rinse a TdT equilibration buffer was placed on the slides for 15 mins. This buffer was blotted off and the TdT label mixture was applied and left to incubate at 37 ºC for 90 mins.

The TdT label mixture contained a mixture of buffer and terminal deoxynucleotide transferase (TdT) enzyme that binds to the exposed 3`-OH ends of DNA fragments found in apoptotic nuclei. Once this mixture was rinsed off the tissue underwent a series of incubations and washes including a ‘stop’ solution for 5 mins, a blocking buffer for 10 mins and a conjugate mixture for 30 mins. Once these were completed and the slides were washed, 3, 3’-diaminobenzidine (DAB) was applied to the tissue for 10 mins and methylgreen was used as a counterstain. All slides were then rinsed, re-hydrated and coverslipped.

2.6.3. Cell Proliferation ki-67 assay

An immunohistochemical assay using the DAKO colour kit (Dako, M7248), along with a monoclonal primary antibody directed against the ki-67 antigen was used to measure the proliferative activity in colonic epithelial cells.

Following the dehydration of tissues, any endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in 50% ethanol for 15 min. Antigen retrieval was required to break the DNA cross links formed by the formalin fixation, so slides were brought to the boil in a citrate buffer (pH 6.5) using a microwave oven, and then left at a low heat for 10 min.

After cooling the tissue sections in the citrate buffer for 30 min, the sections were circled using a PAP pen and incubated with pre block solution for 30 min. Then, the Ki-67 (MIB-5 clone) was applied to tissues at a dilution of 1:1000 in PBS overnight at room temperature. Sections then were rinsed and incubated with a linking agent (poly-HRP anti-mouse IgG) for 30 min and a labelling agent for 30 min and rinsed again. Finally, DAB was applied to all sections for 3 min and was then rinsed under dH_2O. Sections were counter stained with hematoxylin and cells expressing the proliferative antigen Ki-67 had a positively stained brown nucleus.

2.6.4. Short chain fatty acid assay

To obtain the SCFA profiles of solid faecal or caecal matter a simple distillation assay was used that converted each sample into a clean distillate which was then analysed by gas chromatography [166].

Either faecal pellets or caecal contents were collected at a weight of approximately 0.3 -1.0g for each animal. Samples were mixed well with internal standard (1.68mM heptanoic acid, adjusted to 7.0pH using sodium hydroxide) at a volume of 3x the sample weight and placed on ice. The supernatant was then collected from each sample after homogenisation and centrifugation at 3000rpm at 5ºC for 10mins.

A vacuumed distillation manifold complete with several 5ml B1/23 neck Quickfit glass flasks were used for the distillation of all samples. 150µl of sample and 30µl of phosphoric acid was pipetted into each flask and capped immediately. Flasks were swirled in cold ethanol to freeze the sample and then attached to the distillation manifold. By creating a vacuum and directing heat onto each flask using hot water, the vapour from each sample transferred into a clean flask and the resulting distillate was collected and transferred to gas chromatography (GC) vials.

Vials containing the distillate were then placed on a GC machine (Hewlett Packard 5890 series II A) equipped with a flame ionization detector and a capillary column (Zebron ZB-FFAP, 30m x 0.53mmi.d., 1µm film, SGE) and the levels of acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids were all measured to generate a total SCFA profile for each sample. Results are expressed as µmol/g sample.

2.6.5. Long chain fatty acid assay

The LCFA profile of the phospholipid membrane from a variety of tissues was measured using an assay derived from the CSIRO [167]. Samples used were snap frozen in liquid nitrogen and stored at -80°C until ready for use.

To extract the lipid content from samples, 100mg of tissue was frozen in liquid nitrogen and finely ground using a mortar and pestle. Total lipids were extracted from the sample by adding methanol/chloroform/water (2:4:1) and 0.2mmol/L BHT by shaking for 20mins. The suspension was centrifuged for 2mins at 1000 x g and the lower organic layer was transferred to a glass tube and dried down under a stream of nitrogen to eliminate oxidation of lipids.

At this point, the dried down lipids were reconstituted in hexane and plated on a TLC plate (Kieselgel 60 F254, Merck, Germany). The phospholipid content was then separated from the remaining sample by capillary action using a 1:3 acetone/petroleum spirit solvent system containing BHT. The plate was then dried and the origin band was scrapped off and transferred to a capped tube with

1% dry methanol. Following an overnight incubation at 50°C, both petroleum spirit and distilled water were added to each tube and vigorously shaken. The top phase of the mixture was removed and pipetted into a clean glass tube and dried down again under a steam of nitrogen.

After reconstituting the fatty acid methyl esters with hexane, the solution was run through columns containing 20mm Florisil and then eluted with 10% diethyl ether in hexane. Lastly, the solution was then transferred to GC vials, dried down under N_2 again and reconstituted with 50µl isooctane. Samples were then separated and measured by gas chromatography.

2.6.6. ACF methylene blue assay

Colons intended for ACF counting were prepared at the time of kill by being cleaned thoroughly, opened up and stretched out onto Hybond-C protein paper (Amersham Biosciences). They were then fixed in buffered formalin overnight and placed in 70% ethanol.

Prior to staining, colonic tissue was washed briefly in saline and then gently rubbed with a wet swab in order to remove any remaining mucosa. Colonic tissue was rinsed in saline again and then immersed in a 0.1% methylene blue solution for 30 seconds. The entire colon was then examined under a dissecting microscope at 40x magnification with the aid of a grid. Methylene blue stains all colonic tissue and aberrant crypts are clearly identified as, a) being larger than surrounding crypts, and b) having a thicker, more darkly stained crypt opening [168]. The total number of ACF per colon, the size of the ACF and the approximate position of the ACF in the colon were all recorded.

2.7. Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, Illinois). Endpoints were analysed using one way ANOVA (Tukeys), followed by independent t-tests if needed. Difference were considered statistically significant at $p<0.05$.