2001

The effects of R-flurbiprofen in reducing tumors in a multiple intestinal neoplasia mouse model

David Douglas Quiggle

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THE EFFECTS OF R-FLURBIPROFEN IN REDUCING TUMORS IN A
MULTIPLE INTESTINAL NEOPLASIA MOUSE MODEL

A Thesis
Presented to the
Faculty of
California State University
San Bernardino

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
in
Biology

by
David Douglas Quiggle
June 2001
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MULTIPLE INTESTINAL NEOPLASIA MOUSE MODEL

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ABSTRACT

Previous studies have shown that when R-flurbiprofen (R-FB) is administered in the Min/+ mouse model it can cause the prevention and regression of intestinal tumors. To determine the rate and mode of action at which preexisting tumors regress, 72-day old Min/+ mice were administered R-FB or vehicle control for 0, 4, 10, 21 and 42 days. After necropsy tumor loads and apoptotic indices (AI) from normal and tumor appearing tissues were obtained. The data obtained from Min/+ mice administered R-FB demonstrated: 1) a steady decrease in the total number of developed tumors over time; 2) some involvement of increasing the AI in tumor regions, 3) a significant increase of the AI in normal appearing crypts of the small intestine, and 4) no change in the AI of normal appearing villus regions. Increased incidence of apoptosis in the crypts of normal appearing tissue may explain the tumor prevention activity of R-FB. Although there was some evidence of an increased incidence of apoptosis in tumor regions there was not an overall decrease in tumor diameters. Because of this, the possible roles of apoptosis and desquamation in tumor regression remain undefined.
ACKNOWLEDGEMENTS

I would like to thank Encore Pharmaceuticals, Inc. for their support and funding of this study, and to all those who supported me through this long process. Also thanks to Doug Leipold for being my second tumor observer, and Karina Gibson for performing the HPLC work. A special thanks to my family who have stood by me and had to deal with all the time I spent away from them.
To

Staci and Ryan
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CHAPTER ONE

INTRODUCTION

Background

Colon Cancer

In the United States there is an average lifetime risk of over 6% for developing colon cancer which is considered to be high-risk. These statistics place colon cancer as the third most common cancer and the second leading cause of death due to cancer in the U.S. Each year an estimated 150,000 people are diagnosed with colon cancer and 55,000 people die of this disease. In spite of the enormous efforts to improve treatment and develop cures for advanced disease, the overall mortality rates for most forms of epithelial cancers, including colon cancer, have not declined in the past 25 years (Conley, Kaplan and Arbuck 1998 and Hong and Sporn 1997). In light of this information much of current research is focused on genetic, dietary and other risk factors to uncover the predisposition for colorectal cancer in various populations.

Cancers, including colorectal cancer, involve mutated genes that, when expressed, produce molecules that
influence normal cellular activities. Alterations in these normal activities can become devastating if they interfere with the regulation of cellular growth mechanisms. In normal situations, mutations in the genome occur on a regular basis, but are dealt with by mechanisms such as: DNA repair, cell cycle arrest, and/or apoptosis. Although DNA repair and cell cycle arrest play an important part in salvaging a genetically mutated cell, it is apoptosis which acts as the ultimate backup plan by preventing damaged and unwanted cells from replicating. Apoptosis or “programmed cell death” is a morphologically and biochemically distinct form of cell death that can be triggered by a variety of extracellular agents. Apoptosis occurs during normal development as well as in various adult pathophysiological states. Apoptosis plays an important role in organisms as a counterbalance to cellular proliferation (McGill and Fisher 1997). Cellular proliferation is important to form and maintain tissue, but can become harmful if it is not balanced or held in check by apoptosis. The balance between these two processes is vital and the consequences of an imbalance can result in the development of cancer (Foster 2000).
Colorectal cancer arises in a multistep fashion that can take 10-15 years in humans. Accumulating mutations in several genes can result in abnormalities of cellular growth regulation. These genetic alterations transform the normal epithelium into hyperplastic regions, leading to adenoma formation and eventually carcinoma (Winawer 1999 and Marian 1996). During this multistep process in colorectal cancer, cells may develop mutations in the following key genes: the adenomatous polyposis coli (APC), p53 tumor suppressor genes, the Ras oncogene, and various genes that mediate DNA mismatch repair (Shiff and Rigas 1999). The APC gene has been considered the “gatekeeper” of colonic epithelial cell proliferation and inactivation of this gene is required for net cellular proliferation, i.e., imbalance with apoptosis (Umetani et al. 2000). In the intestine, APC protein levels increase at the crypt-villus boundary where cell migration from the crypt is crucial and where cells accumulate during adenoma formation (Nathke et al. 1996). The APC gene is found to be mutated in over 80% of sporadic colorectal adenomas and carcinomas (Tolinson, Ilyas and Novelli 1997). The Ras gene codes for a GTP-binding protein that functions as a signal transducer of extracellular stimuli to regulate cellular proliferation
and differentiation (Finco et al. 1997). The function of the Ras protein is to activate transcription factors (i.e. NF-κB) and pathways that, in return, influence the expression of other genes resulting in overall cellular survival. Oncogenic mutations in Ras, considered to be required for cellular transformation, occur in 50% of colorectal tumors, predominately in larger adenomas and carcinomas (Finco et al. 1997 and Marian 1996). Another gene, p53, also plays a role in cancer development. The normal function of p53 involves sequence-specific DNA binding that promotes cell-cycle arrest or apoptosis in response to a variety of cellular stresses (Lowe 1999). Evidence suggests that mutations in p53 lead to a reduction of apoptosis and rapid tumor progression (Smith et al. 1995). In addition, the gene for p53 is found mutated in 75% of late stage adenomas of colorectal cancer (Toinsom, Ilyas and Novelli 1997 and Marian 1996). Although there are many other genes that are related to colorectal cancer, they are less common than APC, Ras and p53 and their roles in the pathophysiology of colorectal cancer are still under investigation.
Molecular Targets

Characterizing cancers for genetic mutations involved in carcinogenesis and the altered activities they influence have helped researchers discover and/or focus on chemotherapeutic agents that are specific for those molecular targets. In theory, interfering with the activities of genes that are responsible for the loss of cellular regulation should restore the ability of affected cells to undergo apoptosis (Wang et al. 1999). For example, the gene for inducible cyclooxygenase, a key enzyme for the formation of prostaglandins from arachidonic acid, is overexpressed in most gastrointestinal tumors.

There are two known isoforms of the cyclooxygenase enzyme, cyclooxygenase-1 and -2 (COX-1 and COX-2, respectively). The COX-1 enzyme is constitutively expressed and is considered to be responsible for the prostaglandin synthesis required for physiological or "housekeeping" functions of the cell. The COX-2 enzyme, although constitutively expressed in selected tissues, is inducible by a variety of extracellular and intracellular stimuli and is involved in inflammation, mitogenesis and specialized signal transduction (Kam and See 2000). In the gastrointestinal tract, cyclooxygenase-synthesized
prostaglandins, in particular prostacyclin and PGE₂, normally act as cytoprotective agents that regulate gastric acid and mucus production and vasodilation of the gastric mucosa (Kam and See 2000). However, when the COX-2 gene is overexpressed, thereby causing prolonged high levels of prostaglandins, tumor growth may be promoted. In human colorectal carcinomas there is a positive relationship between COX-2 expression and the size, invasiveness and stage of the carcinoma (Fujita et al. 1998 and Sheehan et al. 1999). Although the mechanism for increased COX-2 expression is not completely understood, it is known that oncogenic Ras activates NF-κB, a transcription factor found to be responsible for the increased expression of COX-2. Increased COX-2 then leads to increased prostaglandin synthesis and ultimately inhibition of apoptosis (Mayo et al. 1997 and Kojima et al. 2000). Consequently these steps are considered as early and central events in colon carcinogenesis. Because of the role that COX-2 and NF-κB may have in the progression of colorectal adenomas and carcinomas, they have become important molecular targets for drug development for the prevention and treatment of
Nonsteroidal Anti-Inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin have been used for many years for the treatment of inflammatory diseases such as arthritis. Numerous reports from human epidemiological studies, animal models, and in vitro experiments have all suggested that the administration of NSAIDs represents a potential option in the chemotherapy and chemoprevention of colorectal cancer (Gupta and DuBois 1998). Most NSAIDs are considered COX-inhibitors given that they inhibit both COX-1 and COX-2 to the same degree. However, because these NSAIDs are unable to distinguish between the two COX enzymes, inhibition of COX-1 leads to the blocking of the cytoprotective actions of prostaglandin thus they are implicated in gastrointestinal (GI) toxicities involving gastric mucosal erosions (Wallace et al. 1998). Because traditional NSAIDs exhibit GI toxicities, a new generation of NSAIDs “COX-2 inhibitors” was evaluated for the ability to selectively inhibit the COX-2 enzyme (Fosslien 2000). Although predominately from in vitro and animal studies, evidence indicates that these COX-2 inhibitors have
maintained their anti-neoplastic potential (Kawamori et al. 1998 and Stolina et al. 2000). However, COX-2 inhibitors such as Celecoxib and Vioxx seem to be safer than traditional NSAIDs, though clinical trials have demonstrated that they are not completely free from GI toxicities. Because of the continued GI toxicities, the FDA still requires the manufacturers of the COX-2 inhibitors to carry package warnings similar to the traditional NSAIDs (Feldman and McMahon 2000).

**Alternate Mechanism**

Although there is mounting evidence that COX-2 inhibition plays an important role in the anti-neoplastic effects of NSAIDs, there is also evidence of a second mechanism of action. Studies indicated that COX-2 inhibitors maintain their anti-proliferative activities even in colon cancer cell lines that lack COX-1 and COX-2 expression and therefore lack the ability to produce prostaglandins (Smith, Hawcroft and Hull 2000 and Hanif et al. 1996). While the alternate mechanism is currently unknown it is possible that inactivation of NF-κB could be a candidate.
NF-κB is a ubiquitous, rapid response transcription factor that regulates several classes of genes including those for cytokines, growth factors, other transcription factors and, as previously mentioned, COX-2 (Lee and Burckart 1998 and Kojima et al. 2000). A common theme for all of these NF-κB regulated genes is their involvement with cellular survival. It appears that NF-κB plays a central role in inhibiting apoptosis allowing normal cells to progress towards transformation (Martin, Schmid and Hofer-Warbinek 1999). It has been shown that some of the traditional and COX-2 inhibiting NSAIDs inhibit NF-κB either directly or indirectly (Yamamoto et al. 1999). The ability of NSAIDs to inhibit a transcription factor, such as NF-κB, which has such broad regulatory activities, may be one of the reasons NSAIDs also have many potential uses.

Cyclooxygenase Inhibition

Several classes of NSAIDs are chiral compounds and exist as two enantiomeric forms (R- and S-). Representatives of chiral compounds from the propionic acid class of NSAIDs are ketoprofen, ketorolac, and flurbiprofen. Traditionally, the activity of NSAIDs was assessed based on their ability to inhibit the COX enzymes.
These NSAIDs, in racemic (rac; mixture of both R- and S-enantiomers) form, are potent COX inhibitors and are extensively used as therapeutic agents for anti-inflammatory and analgesic purposes (Carabaza et al. 1996 and Muller et al. 1990). However, when each enantiomer is tested separately it is found that the S-enantiomer is predominately responsible for the COX inhibiting capabilities (Carabaza et al. 1996). Such is the case for Flurbiprofen (FB), the R-enantiomer (R-FB) is considered a non-COX inhibitor due to its high IC$_{50}$ (concentration at which 50% of the enzyme activity is inhibited) for the COX-1 and COX-2 enzymes (400 nM and 1400 nM, respectively). While the S-enantiomer of Flurbiprofen (S-FB) is vastly more potent than R-FB with an IC$_{50}$ for COX-1 and COX-2 of 2.7 nM and 2.5 nM, respectively (Carabaza et al. 1996). S-FB is 148 and 560 times more potent at inhibiting COX-1 and COX-2, respectively, than R-FB, and it was for this reason that R-FB was considered relatively inactive and was labeled as a non-COX inhibiting enantiomer.

Although considered a non-COX inhibitor, R-FB has demonstrated chemoprotective activities in various animal models. In a 30-day study with R- and S-FB (McCracken et
al. 1996), large bowel epithelial cell proliferation rates in laboratory rats were measured by labeling the crypts of Lieberkühn with bromodeoxy uridine (Brd-U) immunostaining. In this study both R- and S-FB at 6.3 mg·kg$^{-1}$·day$^{-1}$ significantly reduced the percent of S-phase cells, when compared to the control animals given vehicle alone (1% CMC, carboxymethylcellulose). This study also demonstrated that S-FB is linked to ulcer formation, which is believed to be due to its COX inhibiting properties. There was an ulcer area approximately 20 times larger with S-FB than with R-FB. Although the ulcer area of R-FB was greater than that found with vehicle control (which exhibited no ulcers), the amount of ulceration was minimal. This minimal ulceration may have been the result of S-FB formation via a process called epimerization or bioinversion, which is an enzymatic conversion of one enantiomer into the other (Wechter 1994). Because rats bioinvert R- to S-FB at a rate of 6 to 10%, there may have been enough S-FB present in these experiments to cause ulceration. However, in human clinical trials with R-FB, the rates of bioinversion to S-FB have been found to be minimal (< 0.5 % bioinversion) at doses greater than
R-Flurbiprofen

The minimal side effects of R-FB clearly give it an advantage over other NSAIDs. To evaluate the effectiveness of R-FB in preventing intestinal adenoma formation, a multiple intestinal neoplasia (Min/+) mouse model was used. The Min/+ mouse is heterozygous for a nonsense mutation in the APC gene and serves as a murine model for the human disease familial adenomatous polyposis (FAP) (Moser, Pitot and Dove, 1990; Su, et al. 1992). Weanling Min/+ mice (30 days old) given doses between 2.5 and 25 mg R-FB·kg⁻¹·day⁻¹ for 6 weeks, exhibited a dose-dependent inhibition (40% to 90%) of adenoma formation (Wechter et al. 1997). In these studies, mice bioinverted R- to S-FB at 6.9% and 16.6% in the 2.5 and 25 mg R-FB·kg⁻¹·day⁻¹ dose groups, respectively. At lower doses (<20 mg·kg⁻¹·day⁻¹) the amount of S-FB was minimal and resulted in little ulceration or other side effects. However, at higher doses (≥20 mg·kg⁻¹·day⁻¹) bioinversion was significant (16.6%) and resulted in increased ulceration. These toxicities resulted in 35% of the animals dying from causes associated
with S-FB toxicity. Because mice exhibit higher R- to S-FB bioinversion rates, S-FB concentrations equivalent to those due to R- to S- bioinversion were tested in the Min/+ mice to determine if the activity of S-FB causes tumor inhibition. This study found that the R-FB inhibition of tumor formation could not be attributed entirely to the R- to S-FB bioinversion (Wechter et al. 1997).

A study that supports the potential use of R-FB as a chemotherapeutic agent for preexisting tumors was performed using the Min/+ mice. In this study the mice were allowed to develop tumors, leaving them untreated until 72 days of age [tumor loads are approximately 25 tumors/mouse in control groups; historical data (Wechter et al. 1997)]. At that point, the mice were treated with 10 mg·kg⁻¹·day⁻¹ R-FB, which had previously proven to be the optimal dose. These mice were treated for six weeks and then intestinal tumors were quantified. There was a significant inhibition (85%) of tumors in the treated group, when compared to the control group (1% CMC). The average tumor load of treated animals were approximately 4 tumors/mouse while the control group had approximately 29 tumors/mouse. This study indicates that R-FB can possibly be used not only as a
chemopreventive agent but also as a chemotherapy to eliminate and reduce existing colon tumors.

In vitro studies involving cancer cell lines and various NSAIDs (i.e. aspirin, naproxen, indomethacin and piroxicam) have displayed an IC$_{50}$ for proliferation ranging between 100 to 600 µM (Shiff et al. 1996). These NSAIDs (with the exception of aspirin) also initiate apoptosis in the tested colon cancer cells (Shiff et al. 1996). To determine if R-FB was also active in vitro, experiments were performed on the colorectal cell line LS174T. In performing proliferation analyses an IC$_{50}$ for proliferation was obtained at approximately 600 µM R-FB. Electrophoretic profiles of DNA from LS174T cells exposed to R-FB for 24 hours revealed DNA laddering, a characteristic of apoptosis (Quiggle, unpublished data). The ability of R-FB to induce apoptosis in vitro, may indicate the method in which it acquires its chemopreventive and chemotherapeutic activities in in vivo studies.

R-flurbiprofen has also been implicated in NF-κB inhibition. In vitro studies involving lipopolysaccharide (LPS) induced NF-κB activation have demonstrated that R-FB inhibits NF-κB activation (Tegeder 2000). This study
demonstrated that R-FB inhibits the LPS-induced nuclear translocation of NF-κB, consequently blocking DNA binding, and inhibiting NF-κB-dependent gene expression. The ability of R-flurbiprofen to inhibit NF-κB may explain the apoptosis-inducing and anti-inflammatory activities associated with this drug.

Study Objectives

Although past studies have demonstrated that R-FB was effective at inhibiting and eliminating colon tumors in the Min/+ mouse model, the rate and mode of eliminating existing tumors remains unknown (Wechter et al. 2000 and Wechter et al. 1997). The purpose of this study was to study the pharmacodynamics of R-FB on tumor regression in Min/+ mice. The following pharmacodynamic parameters were evaluated in response to R-FB treatment: 1) the incidence of apoptosis in the normal appearing crypt and villus regions; 2) the incidence of apoptosis within tumors; and 3) the rate at which the number of tumors decreased. Understanding R-FB pharmacodynamics in reducing tumor number can further test its potential as a chemotherapeutic agent and to aid in the design of future studies by optimizing for length of treatment and timing. Although
understanding the mode by which tumors regress could help support in vitro work conducted with R-FB, it could also help determine potential biomarkers for use in future animal and human studies. Biomarkers from relatively small samples can be used as intermediate indicators of effectiveness in cancer prevention and treatment studies. Biomarkers are especially important in human chemopreventive studies due to the constraints and longevity of human studies.

**Study Design**

The design of the proposed study was to administer R-FB to 72-day old Min/+ mice for up to 42 days. In order to capture the process of tumor reduction, animals were necropsied at various time points. At each time point animals were evaluated for tumor loads and presence of apoptotic cells along the small intestine. Apoptotic cells were assessed using a terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. Apoptotic indexes from the middle to distal section of small intestines were determined in the crypts and villi of normal appearing tissue and of tumor regions.
Study Limitations

A foreseeable limitation of this experiment was the ability to obtain sufficient number of histologic samples of tumor regions in animals treated with R-FB. This was due to the anticipated decrease in the tumor load of the animal. However, to increase the probability of obtaining a histological sample containing a cross-sectioned tumor, samples were cut at several places throughout the evaluated region. This was believed to give a sufficient number of observations in order to be statistically evaluated.
CHAPTER TWO
MATERIALS AND METHODS

Materials

Animals

Fifty-four weanling female C57BL/6J-APC\textsuperscript{Min}/+ mice 26-32 days of age were obtained from Jackson Labs (Bar Harbor, ME). They were housed in the Loma Linda Animal Care Facility with a 12-hour-on:12-hour-off light cycle and controlled for humidity (50\%) and temperature (72°F). The animals were given tap water and pelleted chow (AIN-93G, Dyets, Bethlehem, PA) ad libitum.

Chemicals

R-Flurbiprofen (R-FB) (99\% purity; >99\% enantiomeric excess) was provided by Wycoff Inc. (Lot# 18AZ26C). All other chemicals were commercially available from Fisher Scientific, with purity of reagent grade or better.

Drug Preparation

The R-FB was prepared as a suspension in 1\% carboxymethylcellulose (CMC) by homogenization with a tissue homogenizer, then heating to 60°C for approximately 15 minutes, followed by rehomogenization. The amount of drug prepared was based on a dose volume of 1.0 ml·0.3 kg\textsuperscript{-1}
and the daily dose of 10 mg·kg$^{-1}·$day$^{-1}$ (final concentration of the suspension was 3.0 mg R-FB·ml$^{-1}$ in 1% CMC).

Carboxymethylcellulose (1% CMC) was used as the vehicle control.

Methods

Study Design

R-Flurbiprofen Administration. Upon arrival, AVID microchips (AVID Identification Systems, Inc., Norco, CA) were inserted subcutaneously into the mice for identification purposes. The mice were then randomly assigned into R-FB and vehicle-treated groups (6 mice per group) for each treatment time period (0, 4, 10, 21 and 42 days; with the exception of day-0, which only consisted of a control group) by the Monte Carlo method (Niederreiter 1992). Following randomization, the mice were fed only the AIN-93G diet until 72 days of age. Once aged, treatment began and all mice were administered drug or vehicle on a daily basis, by oral gavage. All mice were weighed weekly and the dosage was recalculated accordingly.

Necropsy. After the completion of the designated treatment periods, the animals were necropsied. Necropsies were performed 2 hours after the animals last administered
dose. At necropsy, the mice were anesthetized by an intraperitoneal (i.p.) injection of 50-mg·kg⁻¹·ketamine and 10-mg·kg⁻¹·xylazine solution. Following the midline abdominal incision, exsanguination via cardiac puncture was performed with a heparinized syringe. From the blood obtained, the hematocrit was determined and a 500 µl aliquot was set aside on ice for determination of plasma drug levels (see below). The animals were then disemboweled between the stomach and rectum and the intestinal tissue was processed as described below.

Analysis of Plasma Drug Levels. Whole blood obtained 2 hours after the last administered treatment was used for determining plasma drug levels. Plasma was obtained by centrifugation (13,000 x g for 5 minutes) of the whole blood. An aliquot of 100 µl was acidified with 20 µl of 2 M HCl and vortexed for 30 seconds. To the acidified solution, 2.0 ml of a ice-cold hexane:ether (4:1) solution was added, followed by 3 minutes of vortexing. The organic and aqueous layers were separated by sonication (to reduce emulsification) and centrifugation (13,000 x g for 15 minutes). A 1.5 ml aliquot of the organic layer was removed and placed into a clean tube and allowed to air dry.
Once dry, the residue was dissolved in 150 µl of a
hexane:isopropanol:acetic acid (97:3:0.5) solution of which
100 µl was analyzed by high performance liquid
chromatography (HPLC) analysis. The HPLC analysis was
conducted on a Beckman System Gold Chromatography System
(version 5.1) running on a PC-compatible computer. The
dissolved plasma residue was applied to a S,S-Whelk-O
column (4.6 x 250 mm; Regis Inc., Morton Grove, IL), and
was eluted with a hexane:isopropanol:acetic acid (97:3:0.5)
solution at 1 ml per minute for 15 minutes. The eluent was
monitored at 254 nm. Standard curves were obtained by
adding rac-FB to 200 µl of mouse whole blood over a range
of 1-40 µg ml⁻¹ of blood. The plasma was analyzed as
described above.

**Determination of Tumor and Ulcer Load.** For
determination of total tumor load, the gastrointestinal
tract was isolated. The three small bowel segments were
excised as the proximal 4 cm (duodenum), the middle 4 cm
(jejunum), and the distal 4 cm (ileum). These sections
were cut longitudinally, washed with PBS (137 mM NaCl, 3 mM
KCl, 4 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.1) and mounted on
bibulous paper with the mucosal side up. These segments
were fixed for approximately 24 hours in 10% neutral
buffered formalin (Fisher cat# HC-200-1 GAL) then transferred into a 70% ethanol (EtOH) solution until analysis.

Tumors and ulcer loads were determined independently by two observers in blind analysis. The segments were soaked in 0.05% aqueous methylene blue (Sigma-Aldrich) for approximately 10 minutes and then viewed with an Olympus SZH dark-field stereomicroscope at a constant magnification (20X). After tumor identification (Figure 1A), the tumor diameter was obtained by measuring the tumor at its widest point. Measurements were taken using an eyepiece reticle that was standardized to a stage micrometer. The smallest measurable tumors were approximately 0.2 mm in diameter. Tumor areas were calculated as: area = \[\pi \left(\frac{\text{diameter}}{2}\right)^2\]. Average tumor area was obtained by taking the average of all tumor areas for each animal then reporting the average of each group. The tumor number reported was the average of the total number of tumors counted from all the segments (proximal, middle and distal) for each animal. Ulcers were identified as non-raised areas, devoid of any villi exposing underlying mucosa, and encircled by tightly bound abnormal appearing villi. The ulcer number reported was
Figure 1. Small intestine tumors and villus stained for apoptosis in C57BL/6J-APC$^{Min}$/+ mice aged to 72 days old then administered R-flurbiprofen orally for Up to 42 days.

the average of the total number of ulcers counted for each individual animal.

Tissue Preparation and Analysis

Tissue Histology. The remaining segment of the middle-distal small bowel was washed with PBS to thoroughly
clean the mucosa of any debris. This section was then cut into 3 to 4 equal portions which were mounted in parallel position on porous tape and rolled up to maintain straight intestinal segments. The intestinal segments were then fixed in 10% neutral buffered formalin for approximately 24 hours and then transferred into a 70% EtOH solution for 24 hours. The segments were dehydrated by incubating in a series of solutions (70% EtOH, 90% EtOH, 95% EtOH, 100% EtOH, xylenes and then a 1:1 xylenes-paraffin solution) for two 30-minute incubation periods in each solution. After the dehydration steps, the segments were embedded longitudinally into paraffin and stored until sectioned.

**Apoptotic Cell Detection.** A microtome (RMC MT-920) was used to cut 4 μm sections from the paraffin embedded intestinal samples. Cut sections were floated in a warm water bath then placed onto aminoalkylsilane treated slides (Polysciences, Inc, Los Angeles, CA), to aid in tissue attachment. Slides were then placed in a 55° C oven for 5 minutes then allowed to cool to room temperature. The slides were then dewaxed and rehydrated by incubating in a series of solutions (xylenes, 100% EtOH, 95% EtOH, 90% EtOH, 70% EtOH and PBS) for two 5-minute incubation periods in each solution. The TUNEL assay was performed
utilizing the TACS™ 2 TdT-DAB In Situ Apoptosis Detection Kit (Travgen Cat # 4810-30K). The tissue samples were then exposed for 30 min to 75-μl of Cytopore™, (saponin-based permeabilization and blocking agent; Trevigen). Each slide was washed twice for 2 minutes in deionized water. Mucosal endogenous peroxidase enzymes were inactivated by soaking the slides in a 3% hydrogen peroxide-methanol solution for 5 minutes, then washing them in PBS for 1 minute. The tissue samples were immersed in a TdT labeling buffer (1M TACS Safe-TdT buffer, 0.5 mg ml⁻¹ BSA, and 0.6 mM 2-mercaptoethanesulfonic acid; Trevigen) for 5-minutes. Excess buffer was removed from the slides by blotting around the tissue samples. A 50 μl volume of labeling reaction mix [5 μM Biotinylated dNTP mix, 1X Mn²⁺ cation solution, TdT enzyme (cat# 4810-30-05; Trevigen)] was added and incubated for 60 minutes at 37°C in a humidified chamber. The reaction was stopped by immersing the slides in a TdT stop buffer (10 mM EDTA, pH 8.0 in deionized water) for 5 minutes and washing each twice in PBS for 2 minutes. Tissue samples were incubated at room temperature for 10-minutes in 50 μl of a Streptavidin-HRP (horseradish peroxidase) solution in PBS, then washed twice in deionized water for 2 minutes each. Each slide was
immersed in DAB solution (Diaminobenzidine, 0.03% hydrogen peroxide, in PBS) for 2 minutes then washed twice in deionized water for 2-minutes. The slides were counterstained with Methyl Green (1.0% Methyl Green in 0.1 M sodium acetate, pH 4.0) for 10 minutes and then dipped 10 times sequentially into deionized water, 95% EtOH, 100% EtOH, and 100% xylenes. The tissue samples were then mounted under glass cover slips using Permount® (Fisher Scientific, cat# SP15-500) mounting media.

Identification and Counting of Apoptotic Cells. An observer (blinded to treatment) counted normal and apoptotic cells from 50 half-crypt (from bottom to top on one side of a crypt) and 50 villus regions (from top to bottom on one side of the villus) for each mouse. A 0.15 mm² area (estimated to contain 3000 cells) was counted for each tumor region (if present up to 10 tumor regions were counted). The apoptotic Index (AI) was defined as the total number of apoptotic cells divided by the total number of cells counted per region, multiplied by 100. Morphological traits such as condensed nuclei and cellular shrinkage were taken into consideration in conjunction with the biochemical assay used to confirm apoptosis. However, in most cases, normal cells were identified by the presence
of a dark blue nucleus and light colored cytoplasm, while apoptotic cells were identified as having a dark brown nucleus and clear to light blue cytoplasm (Figure 1B). Necrotic cells were identified as having a dark brown nucleus and light to dark brown cytoplasm.

Statistical Analyses

Descriptive analyses were applied to find the mean and standard deviation for the represented data. Descriptive statistics were performed on all data using the Kolmogorov-Smirnov Test to test for normal population distribution and Levene Statistic to test for homogeneity of variance (SPSS® Base 9.0 Applications Guide. [1999]). For normal distributed data (all data with the exception of AI for tumor regions, fused villi and ulcer), ANOVA was applied. If the ANOVA test was found to be significant (p ≤ 0.05), the multiple comparisons test of Games-Howell was applied with a significance of p ≤ 0.05 (SPSS® Base 9.0 Applications Guide. [1999]). For nonparametric data (only AI for tumor regions, fused villi and ulcer), the Kruskal-Wallis Test was applied (SPSS® Base 9.0 Applications Guide. [1999]). If this test was found to be significant (p ≤ 0.05), the Mann-Whitney test was applied with a 2-tailed significance of p ≤
0.05. For comparisons not significantly distinguishable by either ANOVA or Kruskal-Wallis, no further analysis was performed. Analyses were performed using SPSS for Windows (Release 9.0.0 [18 Dec 1998]).
CHAPTER THREE

RESULTS

Safety and Tolerance

Dose Tolerability

Overall the mice tolerated the administration of R-FB well. However, as expected signs of anemia (pale-looking muzzle and paws) were observed in the Day-42 vehicle-treated group and this was supported by decreased hematocrits (HCT) measurements taken at necropsy. The vehicle-treated groups showed stable HCT of approximately 35%, up to Day-21 of treatment (93 days of age) relative to the Day-10 vehicle-treated group (Table 1). However, the HCT for the Day-42 vehicle-treated group decreased by approximately 25% when compared to the previous groups. On the other hand, the HCTs for the R-FB treated groups increased throughout most of the study and by day 42 had increased to approximately 40% from the Day-0 vehicle-treated group and approximately 75% from the Day-42 vehicle-treated group (Table 1).

Drug Bioinversion

To determine plasma drug levels in the mice, blood was collected at the approximate Cmax (maximum plasma drug
Table 1. Tolerability of R-Flurbiprofen Administered Orally for up to 42 Days in C57BL/6J-APC<sup>Min</sup>/+ Mice Aged to 72 Days Old.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HCT&lt;sup&gt;‡&lt;/sup&gt; (%&lt;sup&gt;§&lt;/sup&gt;)</th>
<th>% Change B. Wt&lt;sup&gt;‡&lt;/sup&gt; (g)</th>
<th>R-FB&lt;sup&gt;‡&lt;/sup&gt; (µg·ml&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>S-FB&lt;sup&gt;‡&lt;/sup&gt; (µg·ml&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Bioinversion&lt;sup&gt;‡&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>33 ± 8</td>
<td>0 ± 0</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>35 ± 6</td>
<td>2 ± 1</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>R-FB</td>
<td>34 ± 5</td>
<td>0 ± 1</td>
<td>50 ± 19</td>
<td>3 ± 0.2</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td><strong>Day 10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>35 ± 4</td>
<td>1 ± 2</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>R-FB</td>
<td>38 ± 5</td>
<td>2 ± 2</td>
<td>68 ± 11</td>
<td>7 ± 2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td><strong>Day 21</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>36 ± 6</td>
<td>7 ± 2</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>R-FB</td>
<td>43 ± 2</td>
<td>3 ± 3</td>
<td>48 ± 5</td>
<td>3 ± 1</td>
<td>5 ± 2</td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>26 ± 9</td>
<td>6 ± 8</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>R-FB</td>
<td>46 ± 1&lt;sup&gt;†&lt;/sup&gt;</td>
<td>10 ± 3</td>
<td>55 ± 5</td>
<td>3 ± 1</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

<sup>§</sup> All groups contained 6 animals (except Day 42, R-FB treated group had 5 due to 1 death).

<sup>†</sup> P < 0.05 compared to treatment control.

<sup>‡</sup> Values given as mean ± SD.

% Change B. Wt = Percent change in body weight = (necropsy weight - day-0 weight)/day-0 weight·100.

R-FB = R-flurbiprofen.

S-Fb = S-flurbiprofen.

VC = Vehicle Control (1% carboxymethylcellulose).

HCT = Hematocrit.

ND = None Detected.

Bioinversion = S-FB(µg·ml<sup>−1</sup>)/[R-FB (µg·ml<sup>−1</sup>)+ S-FB (µg ml<sup>−1</sup>)].
concentration) of R-FB. Overall the levels of R-FB were within previously reported values (Wechter et al. 1997). Because mice are a bioinverting species, concentrations of S-FB between 3 and 7 μg ml⁻¹ correspond to approximately 5 to 9% bioinversion (Table 1).

Animal Deaths

One death occurred 35 days into treatment in an animal scheduled to be treated with R-FB for 42 days (Table 1). Prior to death, the animal demonstrated normal behavior, weight gain, and no physical signs of morbidity. This animal was found dead in its cage probably due to iatrogenic effects during the administration of oral gavage.

Pharmacodynamics

Tumor Evaluation

Tumor loads for individual animals decreased with time in the R-FB treated animals. The R-FB treated groups had fewer total tumor numbers after 10 days of treatment and by days 21 and 42 of treatment the tumors number was significantly less by approximately 70% and 87% (p ≤ 0.05), respectively (Table 2). In the control animals, the tumor
Table 2. Tumor Counts Evaluated in C57BL/6J-APC<sup>Min</sup>/+ Mice Aged to 72 Days Old then Administered R-Flurbiprofen Orally for Up to 42 Days.

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;$&lt;/sup&gt;</th>
<th>Tumor #&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Tumor Diameter&lt;sup&gt;+&lt;/sup&gt; (mm)</th>
<th>Tumor Area&lt;sup&gt;+&lt;/sup&gt; (mm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>24 ± 18</td>
<td>0.7 ± 0.13</td>
<td>0.5 ± 0.17</td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>26 ± 18</td>
<td>0.7 ± 0.13</td>
<td>0.6 ± 0.24</td>
</tr>
<tr>
<td>R-FB</td>
<td>27 ± 8</td>
<td>0.5 ± 0.07</td>
<td>0.3 ± 0.11</td>
</tr>
<tr>
<td><strong>Day 10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>26 ± 16</td>
<td>0.7 ± 0.10</td>
<td>0.6 ± 0.17</td>
</tr>
<tr>
<td>R-FB</td>
<td>16 ± 7</td>
<td>0.5 ± 0.07&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.2 ± 0.08&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Day 21</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>25 ± 7</td>
<td>0.8 ± 0.08</td>
<td>0.8 ± 0.10</td>
</tr>
<tr>
<td>R-FB</td>
<td>7 ± 2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.5 ± 0.14&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.2 ± 0.15&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>27 ± 10</td>
<td>1.0 ± 0.21</td>
<td>1.2 ± 0.49</td>
</tr>
<tr>
<td>R-FB</td>
<td>4 ± 2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.4 ± 0.25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.2 ± 0.24&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>$</sup> All groups contained 6 animals (except Day 42, R-FB treated group had 5 due to 1 death).
<sup>+</sup> P ≤ 0.05 compared to treatment control.
<sup>+</sup> Values given as mean ± SD.
VC = Vehicle Control (1% CMC).
R-FB = R-flurbiprofen.

Number remained stable at approximately 25 tumors per animal for all treatment periods.

Treatment also influenced the size of the tumor. Tumors from the Day-4 R-FB treated groups decreased in the average tumor diameter by about 25% compared to the Day 0 control group (0.5 ± 0.07 mm vs. 0.7 ± 0.13 mm), and maintained an average diameter of approximately 0.5 mm.
throughout the 42 days of treatment. The tumor diameters of the vehicle-treated groups grew approximately 25% by day 42 of treatment (0.75 mm ± 0.13 at day 0 to 1.05 mm ± 0.21 at day 42). Since the tumor diameters were less in the R-FB treated animals and greater in the vehicle-treated groups, a significant difference (p ≤ 0.05) of approximately 25% was established by Day-10 of treatment and had increased to 38% and 55% by 21 and 42 days of treatment, respectively. Analysis of the data obtained from both observers revealed no significant inter-observer variance. Gastrointestinal observations described as ulcers were found in the early portion of this study (Table 3). These ulcers were found predominantly in the R-FB treatment groups, with the majority occurring at Day-10 of treatment and none being found at Day-42 of treatment. No ulcers were found in the vehicle-treated groups, with the exception of one animal with one ulcer. Fused villi were found only in the treated groups, with the exception of one animal in the Day-10 vehicle-treated group. The Day-21 R-FB treated group contained the most fused villi, while the Day-4 treated group had none. However, fused villi
Table 3. Small Intestinal Observations in C57BL/6J-APC\textsuperscript{Min}/+ Mice Aged to 72 Days Old then Administered R-Flurbiprofen Orally for Up to 42 Days.

<table>
<thead>
<tr>
<th>Treatment\textsuperscript{§}</th>
<th>Fused Villi\textsuperscript{‡}</th>
<th>Ulcers\textsuperscript{‡}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>N.O.</td>
<td>N.O.</td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>N.O.</td>
<td>N.O.</td>
</tr>
<tr>
<td>R-FB</td>
<td>N.O.</td>
<td>2 ± 2\textsuperscript{†}</td>
</tr>
<tr>
<td><strong>Day 10</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>0.3 ± 0.8</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>R-FB</td>
<td>3 ± 3\textsuperscript{†}</td>
<td>4 ± 3\textsuperscript{†}</td>
</tr>
<tr>
<td><strong>Day 21</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>N.O.</td>
<td>N.O.</td>
</tr>
<tr>
<td>R-FB</td>
<td>7 ± 3\textsuperscript{†}</td>
<td>2 ± 2\textsuperscript{†}</td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>N.O.</td>
<td>N.O.</td>
</tr>
<tr>
<td>R-FB</td>
<td>5 ± 4\textsuperscript{†}</td>
<td>N.O.</td>
</tr>
</tbody>
</table>

\textsuperscript{§} All groups contained 6 animals (except Day-42, R-FB treated group had 5 due to 1 death).

\textsuperscript{†} P \leq 0.05 compared to treatment control.

\textsuperscript{‡} Values given as mean ± SD.

N.O. = No Observations.
VC = Vehicle Control (1% CMC).
R-FB = R-flurbiprofen.

 were found from day 10 through day 42 in the R-FB treated groups.

**Apoptotic Index**

When R-FB was administered, the apoptotic index for the crypts of Lieberkuhn sharply increased (Table 4). A significant two- to three-fold increase in the crypt apoptotic index was observed in the R-FB treated groups.
Table 4. Apoptotic Index and Cell Positions of the Crypt, Villus and Tumor Regions in C57BL/6J-APC<sup>Min</sup>/+ Mice Aged to 72 Days Old then Administered R-Flurbiprofen Orally for Up to 42 Days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length (days)</th>
<th>N</th>
<th>Apoptotic Index (%)</th>
<th># Cells/ Half-Region&lt;sup&gt;‡&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crypt Region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>6</td>
<td>1.08 ±0.93</td>
<td>13.6 ±0.8</td>
</tr>
<tr>
<td>VC</td>
<td>4</td>
<td>6</td>
<td>0.71 ±0.51</td>
<td>13.8 ±1.0</td>
</tr>
<tr>
<td>R-FB</td>
<td>4</td>
<td>6</td>
<td>2.70 ±0.57&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>14.1 ±0.5</td>
</tr>
<tr>
<td>VC</td>
<td>10</td>
<td>4</td>
<td>1.30 ±0.50</td>
<td>12.7 ±0.2</td>
</tr>
<tr>
<td>R-FB</td>
<td>10</td>
<td>1</td>
<td>3.73</td>
<td>-</td>
</tr>
<tr>
<td>VC</td>
<td>21</td>
<td>6</td>
<td>0.55 ±0.45</td>
<td>14.2 ±0.4</td>
</tr>
<tr>
<td>R-FB</td>
<td>21</td>
<td>6</td>
<td>1.98 ±0.68&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>13.0 ±0.8</td>
</tr>
<tr>
<td>VC</td>
<td>42</td>
<td>6</td>
<td>0.84 ±0.79</td>
<td>13.5 ±0.8</td>
</tr>
<tr>
<td>R-FB</td>
<td>42</td>
<td>5</td>
<td>2.90 ±0.32&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>12.8 ±0.6</td>
</tr>
<tr>
<td><strong>Villus Region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>6</td>
<td>0.39 ±0.29</td>
<td>34.1 ±1.7</td>
</tr>
<tr>
<td>VC</td>
<td>4</td>
<td>6</td>
<td>0.23 ±0.12</td>
<td>35.4 ±1.9</td>
</tr>
<tr>
<td>R-FB</td>
<td>4</td>
<td>6</td>
<td>0.30 ±0.17</td>
<td>34.9 ±2.1</td>
</tr>
<tr>
<td>VC</td>
<td>10</td>
<td>4</td>
<td>0.22 ±0.24</td>
<td>35.6 ±3.8</td>
</tr>
<tr>
<td>R-FB</td>
<td>10</td>
<td>1</td>
<td>0.22</td>
<td>-</td>
</tr>
<tr>
<td>VC</td>
<td>21</td>
<td>6</td>
<td>0.27 ±0.20</td>
<td>35.8 ±2.3</td>
</tr>
<tr>
<td>R-FB</td>
<td>21</td>
<td>6</td>
<td>0.95 ±0.51</td>
<td>31.0 ±1.6&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>VC</td>
<td>42</td>
<td>6</td>
<td>0.39 ±0.28</td>
<td>34.8 ±2.8</td>
</tr>
<tr>
<td>R-FB</td>
<td>42</td>
<td>5</td>
<td>0.85 ±0.53</td>
<td>33.4 ±2.5</td>
</tr>
</tbody>
</table>

| **Tumor Region** | | | | |
| None      | 0             | 5 | 0.11 ±0.06         |                               |
| VC        | 4             | 5 | 0.08 ±0.02         |                               |
| R-FB      | 4             | 6 | 0.12              | -                             |
| VC        | 10            | 3 | 0.12 ±0.04         |                               |
| R-FB      | 10            | 6 | 0.12              | -                             |
| VC        | 21            | 6 | 0.08 ±0.03         |                               |
| R-FB      | 21            | 6 | 0.29 ±0.22         |                               |
| VC        | 42            | 6 | 0.08 ±0.03         |                               |
| R-FB      | 42            | 6 | 0.52 ±0.58         |                               |

<sup>†</sup> P < 0.05 compared to day 0 control.<br><sup>‡</sup> P < 0.05 compared to treatment control.<br>Values given as mean ± SD.<br>VC = Vehicle Control (1% CMC). N = Sample number<br>R-FB = R-flurbiprofen.<br>Apoptotic Index = # apoptotic cell/# normal cells + # apoptotic cells) × 100.
compared to the vehicle-treated groups and was maintained throughout the study. However the increase in the incidence of apoptotic cells did not result in a change in the number of cell positions lining the half-crypt (Table 4).

The apoptotic index of the villus region did not match that of the crypt region. There was no difference in the apoptotic index between the R-FB treated groups and the vehicle-treated groups. The number of cell positions lining the half-villus also remained unchanged between the vehicle and R-FB treated groups, with the exception of the Day-21 groups. The Day-21 R-FB treatment group showed an apoptotic index decrease of approximately 13% compared to the vehicle-treated group. However, this difference was not easily explained relative to the other R-FB treated groups, since they did not demonstrate any change in the apoptotic index.

The apoptotic index of tumor regions proved to be a difficult measurement to obtain. Because of the spacing of tumors and the reduction of tumor number in R-FB treated animals, the task of obtaining a slide section that bisects a tumor was very difficult and influenced the number of animals that were evaluated for tumor AI. Although there
appeared to be a large increase in the apoptotic index of Day-21 and Day-42 treated groups, the small and unequal sample size severely affected statistical validation of comparisons and prevented its analysis.

The multi-step process to prepare the samples for TUNEL was vital to properly assess for apoptotic cells. For an unidentified reason, the majority of the Day-10 animals could not be assessed because the paraffin embedded tissue samples were either too fragile or brittle and were unable to withstand the TUNEL assay procedures.
CHAPTER FOUR
DISCUSSION AND CONCLUSION

Discussion

Previous studies have shown that when R-flurbiprofen was administered in the Min/+ mouse model it caused the prevention and regression of Min/+ tumors. This study addresses the issue of the rate at which these tumors regress and mode of action. The data obtained from this study demonstrate that R-flurbiprofen administration in the Min/+ mouse model caused: 1) a time-dependent decrease in the total number of developed tumors; 2) some involvement of increasing apoptosis in tumor regions, 3) a significant increase of the AI in normal appearing crypts of the small intestine, and 4) no change in the AI of normal appearing villus regions. Previous studies involving other NSAIDs have demonstrated rapid regression of tumors and increased AI in only the villus region (Boolbol et al. 1996, Chiu, McEntee and Whelan 1997 and Mahmoud et al. 1998). Although this study was contradictory regarding the AI incidence, these results seem to be more rational based on GI epithelial cellular kinetics.
Previous studies with R-FB in the Min/+ mouse model found approximately 70% to 90% tumor inhibition after 42 to 204 days of treatment (Wechter et al. 1997 and Wechter et al. 2000). In the current study, tumors decreased in number gradually beginning between days 4 and 10 of drug administration and continuing through day 42. The tumor regression observed was not as rapid as those exhibited in a similar study using sulindac, a potent COX-2 inhibitor of the acetic acid class of NSAID (Chiu, McEntee and Whelan 1997). In the sulindac study, tumor loads were evaluated for up to 20 days, and displayed a rapid regression of tumor number. There was a rapid time-dependent decrease in the tumor load of the sulindac treated animals. However, due to weak inhibition by R-FB of the COX enzymes, it was anticipated that a slower regression would have occurred.

The assumption in this study was that if R-FB affects tumor growth by stimulating apoptosis, the apoptotic index (AI) would increase in the tumor regions and slowly decrease the tumor size until the tumor was no longer present. Obtaining an AI for the tumor regions in all animals proved to be a difficult task. The AI’s that were obtained were based on a small number of animals which were not large enough to perform statistical analyses. However,
as hypothesized the average tumor diameter did not decrease over time but actually stayed relatively stable throughout the study in the R-FB treated animals (Table 2). This may indicate that the loss of single tumors happens over a relatively short time frame. One method of rapid tumor loss is when a whole tumor detaches from the wall of the intestine and is lost into the lumen of the intestine (desquamation). This concept was considered in the sulindac study mentioned above. Because of the rapid decrease in tumor counts and the inability to show an increase in AI in the tumor regions, however those authors also had difficulties with obtaining AI’s (Chiu, McEntee and Whelan 1997). One piece of evidence that suggests that desquamation may occur was one histological slide of the small intestine of an R-FB treated animal (Figure 2). The arrow points to a large mass of tissue within the small intestinal lumen with a large number (approximately 80%) of the cells undergoing apoptosis or necrosis. This observation was seen over several serial sections and was believed not to be an artifact of preparation. If these tumors did in fact undergo desquamation, this rapid process may leave behind an ulcer-like mucosa.
Figure 2. Small intestinal lumen from a C57BL/6J-APC\textsuperscript{Min}/+ mouse administered R-flurbiprofen orally.

The observations of ulcers in this study were first thought to be the result of ulcer induction due to the small amount of bioinversion from R-FB to S-FB present in this model. However, it seems that the ulcer appearance may correlate better with tumor loss. The data show that over time, ulcers appear first during times of more rapid
tumor regression and they are absent at day 42, a time when tumor regression rate may be at its lowest. This correlation may indicate that desquamation of the tumors occurs, leaving behind a region with ulcer-like appearance. The observation of fused villi was believed to be the result of ulcers healing and this may still be the case. Fused villi appeared after the presence of ulcers and were then present throughout the rest of the study. Although the sulindac study (Chiu, McEntee and Whelan 1997) had mentioned "crateriform lesions" (believed to be ulcers as described by this paper), they had never suggested that desquamation may be the cause. Although this study was not designed to study the correlation between tumor regression, ulcer formation and fusion of villi these results warrant further analysis and should be the subject of additional studies.

The AI was studied in the crypt and villus regions of normal appearing tissues. Although the villus showed no increase in AI over time, only the crypt region had a significant increase in AI throughout the study. This was the opposite of what was seen in the sulindac studies (Boolbol et al. 1996, Chiu, McEntee and Whelan 1997 and Mahmoud et al. 1998), which reported the AI increases only
in the top portion of the villus region. In the present study the AI of the crypt regions increased 2- to 5-fold more than the vehicle-treated groups. These results are favorable, based on stem cell kinetics of the small intestine. These stem cells reside in the crypt of the small intestine. The number of stem cells has a positive influence on the proliferation rate of the gastrointestinal epithelium. Studies suggest that the number of stem cells and the apoptosis rate are vital in maintaining constant cell populations in of the gastrointestinal epithelium (Potten, Wilson and Booth 1997 and Renehan, Bach and Potten 2001). This is because one stem cell can produce hundreds of cells that migrate up the crypt and onto the villus. When the number of stem cells goes unchecked by apoptosis, tumorigenesis can occur (Renehan, Bach and Potten 2001). Therefore, the ability of R-FB to increase the incidence of apoptosis in the crypt opposed to the villus, gives greater support to its anti-tumor activities and as a potential therapeutic agent.

As with any drug, safety and tolerance are important to the success of an efficacious chemotherapeutic agent. The current study has shown that those animals treated with R-FB for 42 days had improved hematocrits from that of Day-
0 and Day-42 vehicle-treated animals. Hematocrits have proven to be an important surrogate marker for tumor burden in the Min/+ mice. It is believed that as the tumor load increases, hematocrit is adversely affected over time. The one death that was observed in this study was believed to be iatrogenic in origin possibly during the administration of oral gavage. Based on previous experience this is a rare yet expected consequence of drug administration by the use of oral gavage, especially in cases when no adverse observations were noted prior to the animal’s death. Based on the current study, along with human studies, it is further supported that R-FB is a safe and well tolerated drug to administer as a chemotherapeutic agent (Quiggle et al. 2000).

Conclusion

In conclusion, the data obtained from Min/+ mice administered R-flurbiprofen, demonstrated: 1) a steady decrease in the total number of developed tumors over time; 2) some involvement of increasing the AI in tumor regions, 3) a significant increase of the AI in normal appearing crypts of the small intestine, and 4) no change in the AI of normal appearing villus regions. Increased incidence of
apoptosis in the crypts of normal appearing tissue supports the tumor prevention activity of R-FB. Although there was some evidence of an increased incidence of apoptosis in tumor regions there was not an overall decrease in tumor diameters. Because of this, the possible roles of apoptosis and desquamation in tumor regression are still unknown. With a better understanding of the rate at which tumors decrease in number with R-FB administration, future studies can be better designed to optimize the time frame in which to capture the process of tumor regression.
REFERENCES


