CHEMO-PREVENTATIVE EFFECTS OF HYDROGEN SULFIDE-RELEASING NSAIDS IN MURINE COLORECTAL CANCER

McMaster University

CHEMO-PREVENTATIVE EFFECTS OF HYDROGEN SULFIDE-RELEASING
NSAIDS IN MURINE COLORECTAL CANCER

By

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ABSTRACT

Colorectal cancer leads to more than 600,000 deaths worldwide per year. An abundance of research has shown that several non-steroidal anti-inflammatory drugs (NSAIDs) can exert chemotherapeutic and chemo-preventative effects in colorectal cancer patients. It is important to note, that use of many different NSAIDs carries a significant risk for cardiovascular and gastrointestinal (GI) complications. A recently developed group of NSAIDs, which release hydrogen sulfide (H_2S), has been shown to have greatly reduced these side effects as compared to conventional NSAIDs. This is likely attributable to the ability of H_2S to increase the resistance of the GI mucosa to injury, as well as to accelerate repair of injury when it occurs. Moreover, H_2S has been shown to be a vasodilator, and therefore may offset some of the hypertensive effects of NSAIDs.

We assessed the chemotherapeutic actions of two of these newly developed NSAIDs. ATB-346 is an H_2S-releasing derivative of naproxen and ATB-352 is an H_2S-releasing derivative of ketoprofen. These drugs were tested in the azoxymethane mouse model and in the $APC^{Min/+}$ mouse model of Colorectal cancer.

In the azoxymethane model of colorectal cancer ATB-346 caused a significant reduction in number aberrant crypt foci (ACF), which are pre-neoplastic lesions used as markers of colorectal cancer. The reduction was superior to naproxen at all doses tested. ATB-352 also caused a significant reduction in the number of ACF, however the reduction was not superior to that produced by ketoprofen. In $APC^{Min/+}$ mice treated with ATB-346 for 14 days (14.5 mg/kg) we observed a complete inhibition of the formation of colonic polyps/tumours and a 97.5% reduction in total polyp score. Shorter treatment with ATB-346 also produced similar reduction in total polyp score. We found that ATB-346-treated

mice had lower levels of β-catenin and cmyc without significant changes in APC or p53 levels.

These results demonstrate ATB-346 can exert superior chemo-preventative effects in mice models of colon cancer while leading to no gastric or intestinal damage.

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Finally, I would like to thank my friends and family, primarily for reading and editing this but also for their continuous encouragement and for supporting me in the pursuit of my dreams. I would specifically like to acknowledge my parents who taught me the value of hard work and an education may help you accomplish anything you want.

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LIST OF ABBREVATIONS

3MP	3-mercaptopyruvate
3MST	3-mercaptopyruvate sulfurtransferase
APC	Adenomatous Polyposis Coli
ACF	Aberrant Crypt Foci
ATB-346	[2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl phenylester]
CAT	Cysteine aminotransferase
CBS	Cystathionine-β-synthase
CMC	Carboxymethylcellulose
CO	Carbon monoxide
Cox	Cyclooxygenase
CSE	Cystathionine-γ-lyase
DADS	Diallyl Disulfide
DMSO	Dimethylsulphoxide
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunosorbent assay
FAP	Familial Adenomtous Polyposis
GI	Gastrointestinal
GSK-3β	Glycogen Synthase Kinase 3β
H_2S	Hydrogen sulfide
IFN-γ	Interferon-gamma
IL	Interleukin
i.p.	Intraperitoneally
Keap1	Kelch-like ECH-Associated Protein 1
MPO	Myeloperoxidase
NaHS	Sodium Hydrosulfide
NaS	Sodium Sulfide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NO	Nitric oxide
Nrf2	Nuclear Factor (erythriod-derived 2)- Like 2
NSAID	Non-steroidal anti-inflammatory drug
NQO1	NAD(P)H Dehydrogenase, Quinone 1
p53BP1	p53 Binding Protein 1
P5P	Pyridoxal-5'-phosphate
PG	Prostaglandin
PGG/HS	Prostaglandin endoperoxide G/H synthases
PGI$_2$	Prostacyclin
PO	Per Os (Oral Administration)
TBZ	4-hydroxythiobenzamide
TNF-α	Tumor Necrosis Factor-alpha
TXA$_2$	Thromboxane A$_2$
WT	Wild type

DECLARATION OF ACADEMIC ACHEIVEMENT

Experiments were conceived and designed by Wagdi Elsheikh and Dr. John Wallace. Wagdi Elsheikh performed experiments with assistance from Dr. Terence Agbor, Webb McKnight and Kyle Flannigan. Wagdi Elsheikh and Dr. John Wallace performed data analysis. Wagdi Elsheikh wrote this thesis.

1.0 GENERAL INTRODUCTION

1.1. Colorectal Carcinoma

Colorectal cancer is the fourth leading cause of cancer death worldwide with over 600,000 deaths each year (Ferlay et al., 2010). Although extraordinary accomplishments have been made in the detection, diagnosis and discovery of specific molecular mechanisms of colorectal cancer there is currently no cure for this disease. The predominant form of colorectal cancer is sporadic with no familial association (Itzkowitz & Yio, 2004), though the hereditary form of colorectal cancer, known as Familial Associated Polyposis (FAP), accounts for a small percentage of colorectal cancer. Of all colorectal cancer, 70% of sporadic colorectal cancers are due to bi-allelic inactivation of the Adenomatous Polyposis Coli (APC) gene. APC is a protein involved in the Wnt- β - catenin signaling pathway. Mutations in this signaling pathway are the only known genetic alterations present in early premalignant lesions in the intestines, such as aberrant crypt foci (ACF) and small adenomas or polyps. Constitutive activation of the Wnt signaling pathway caused by mutations in components of the pathway is responsible for the initiation of colorectal cancer (Mantovani et al., 2008).

Colorectal cancer originates in the epithelial cells that line the colon or rectum. These cells begin to hyper proliferate and become dysplastic. As previously stated, the initiation of colorectal cancer stems from an over-activation mutation in the Wnt pathway (Hanahan & Weinberg, 2011). There are several identifiable steps in the course of colorectal carcinogenesis. First, normal epithelial cells with a mutation in one of the proteins in the Wnt pathway, or any other pro-oncogenes may become non-apoptotic. In the Wnt pathway, the Wnt ligand is a glycoprotein that binds to the frizzled receptors.

After binding, the complex attaches to a co-receptor, LRP5/6, leading to phosphorylation and activation of the intracellular kinase Dishevelled (Dvl). Activation of Dvl displaces GSK-3β from its complex with APC and Axin. The APC/Axin/GSK-3β is a complex that regulates β-catenin. In the presence of the Wnt ligand, β-catenin is not degraded, allowing β-catenin to translocate into the nucleus. Nuclear β-catenin has been reported to activate the expression of a number of genes, including cmyc, cyclin D1, and MMP-7 (Hanahan & Weinberg, 2011; Morin, 1997). Due to these mutations precancerous epithelial cells gradually inhibit apoptosis. The cells then lose their activity of tumor suppressor genes (i.e./ APC, p53, K-ras etc.) and acquire oncogenic mutations (i.e. β-catenin,) promoting the progression to carcinoma (Bird, 1995; Pretlow, 2005).
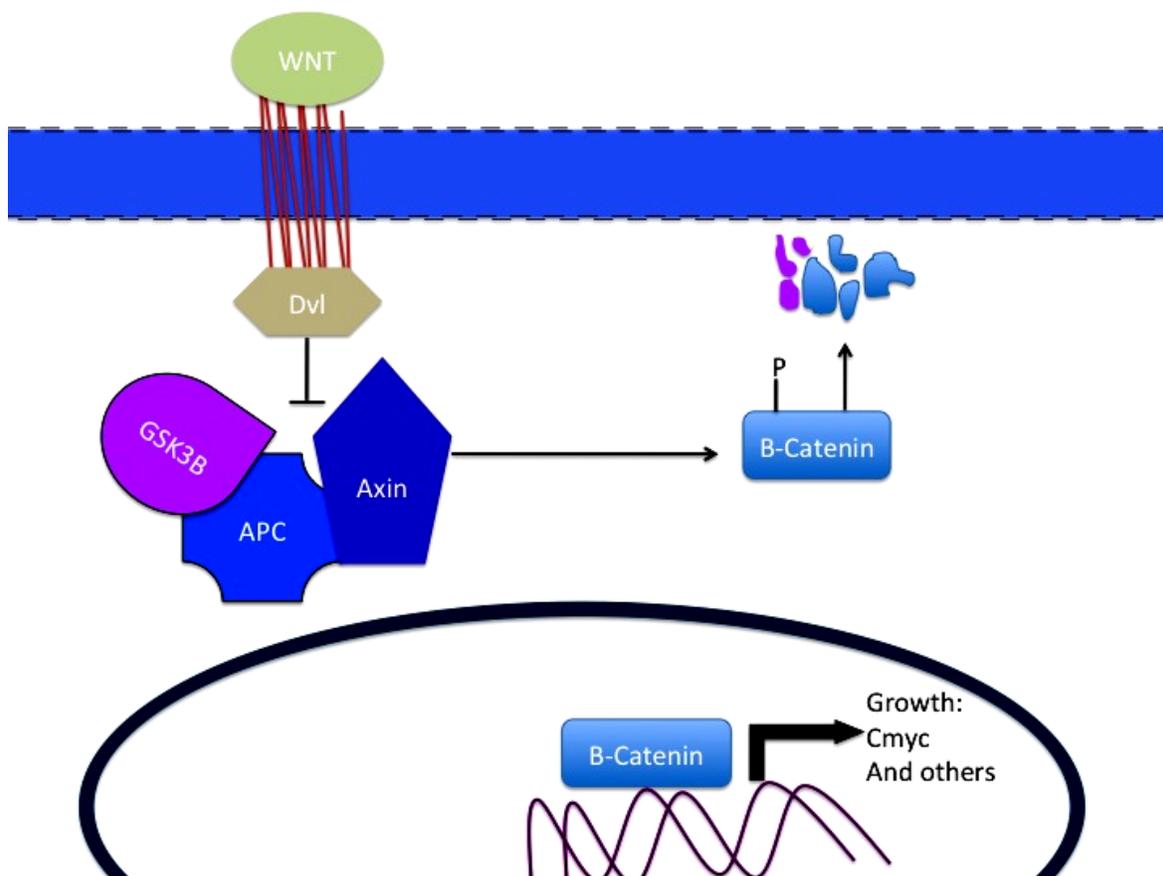
Figure 1.0: The Wnt signaling pathway. When Wnt ligand binds to the frizzled ligand leading to β-catenin's translocation into the nucleus and the consequential transcription of proteins that lead to cell proliferation. Figure adapted from

1.2. Models of Colorectal Carcinoma

The animal models used to study colorectal cancer involve activating mutations in either the Wnt pathway or other related oncogenic pathways to initiate tumorogenesis in the intestine. The mutations induced in these models are similar to the disease process found in humans, which is characterized by the formation of dysplastic crypts and adenomas. The two predominant animal models of colorectal cancer induce mutations in the Wnt-β-catenin pathway leading to the formation of colorectal cancer in rodents. The first model involves the use of azoxymethane, a potent carcinogen that induces colon cancer in rodents similar in pathogenesis to human sporadic colorectal cancer. Once administrated, azoxymethane is metabolized into methylazoxymethanol by CYP2E1, which causes the DNA mutations leading to colorectal cancer (Ward et al., 1973). Most commonly, mutations lead to the over activation of β-catenin (Erdman et al., 1997). Mutations can also occur in the regulation of β-catenin or in its regulators (the APC/Axin/GSK-3β complex), preventing the degradation of β-catenin (Narisawa et al., 1976).

The second model of colorectal cancer uses heterozygous C57BL/6- Apc$^{Min/+}$ mice (Apc$^{Min/+}$). The Apc$^{Min/+}$ mouse strain contain a mutation in the murine *APC* gene that predisposes them to develop multiple intestinal adenomas (Moser et al., 2011) and is similar to humans suffering from FAP as they also carry a mutation in the *APC* gene.

$Apc^{Min/+}$ mice develop tubular adenomas primarily in the intestine and, less frequently, in the colon. These mice die within 169 days of age (Takahashi et al., 2000).

These two models have been used extensively to research possible treatments and preventatives for colorectal cancer.

1.3. Non Steroidal Anti-Inflammatory Drugs

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are a class of drugs that produce antipyretic, analgesic and anti-inflammatory effects within the body. NSAIDs are commonly prescribed to patients suffering from pain due to arthritis, menstrual cramps, gout and headaches (Stolfi et al., 2013).

NSAIDs produce anti-inflammatory effects by inhibiting the enzymatic synthesis of prostaglandins (PGs). The majority of NSAIDs inhibit PG production by acting as reversible competitive inhibitor of Cox activity (Moncada et al., 1975). Cox-1 and Cox-2 are expressed in a variety of tissues. Cox-2 is rapidly increased during inflammation by inflammatory cytokines (Collier, 1971; Vane & Botting, 1998; Vane, 1976). However, this theory is currently under question because Cox-1 has also been up-regulated in inflammation (Wallace, 2007). In order to understand the mechanism of NSAID action a brief description of the PG synthesis is necessary. When a cell is activated by cytokines, growth factors or mechanical trauma it triggers the activation of phospholipases. The phospholipases release arachidonic acid from membrane lipids. Once released, Cox-1 and Cox-2 metabolize arachidonic acid into $PGGH_2,$ then PGH_2. A cell-type specific enzyme then metabolizes PGH_2 to biologically active prostanoids that may act to mediate a number of physiological responses.

PGs have several diverse functions within the body and elicit effects often associated with inflammation and cancer. They are a group of bioactive lipids that include PGE_2, $PGF_2\alpha$, PGD_2, PGI_2 and TXA_2 (Wright, 1993). PGs act as autocrines or paracrines and may induce contraction and relaxation of smooth muscle tissue, regulate cell growth, induce labor, cause platelet aggregation may inhibit stomach acid secretion, induce fever, sensitize neurons to pain and control several inflammatory mediators (Nakanishi & Rosenberg, 2013). In addition, PGs not only induce inflammation they also mediate gastro duodenal mucosal protection and maintain mucosal homeostasis (Wallace, 2008). PGE_2 is the major PG involved in protection of mucosa, and in responses to tissue injury it induces vasodilatation, an influx of innate immune cells, edema, increased pain response by stimulating sensory nerves, and promotes pyrogenic effects (Wallace, 2008). PGE_2 is involved in the resolution of inflammation and tissue repair. It also has been shown as an immunosuppressant by directly inhibiting synthesis of interleukin-2 (IL-2) and the expression of IL-2 receptors. This leads to decreased activation and proliferation of T cells, $\gamma\delta$-Tcells and natural killer (NK) cells (Nakanishi & Rosenberg, 2013). PGE_2 has also been shown to inhibit the production of CCL19, a chemokine produced by monocytes and dendritic cells to attract naïve T-cells to the site of inflammation. PGE_2 has been shown to skew the T cell response to a TH2 cell response, and with greater TH2 response there is a reduction in inflammation and an increase in resolution (Robert, 1984). In addition to its immunosuppressive functions, PGE_2 has been shown to induce wound repair by directly inducing epithelial cell proliferation (Wallace & Granger, 1996).

Two commonly prescribed NSAIDs are ketoprofen and naproxen. Ketoprofen is a propionic acid class of NSAID commonly prescribed for rheumatic disease and pain and is

a selective Cox-1 inhibitor (Lobetti & Joubert, 2000). Naproxen is in the arylacetic acid class of NSAIDs commonly prescribed for rheumatic disease, inflammation and pain. It is an inhibitor of Cox-1 and Cox-2. Although the mechanism of action of most NSAIDs is similar, their pharmacokinetic properties and elimination pathways vastly differ (KuKanich et al., 2012). Factors such as half-life, concentration in serum and metabolic activation greatly influence their anti-inflammatory variation between each NSAID (Mantovani et al., 2008).

1.4. The Role of PGE_2 in Cancer

The association between chronic inflammation and development of cancer is well recorded (Rigas et al., 1993). However, the specific mechanism that leads to cancer is not as clearly understood. In particular, cases of inflammatory bowel disease have been directly linked with a significant risk factor for colorectal cancer (Mantovani et al., 2008). It has been previously described that PGE_2 plays an essential role in the progression of inflammation-related intestinal cancers (Trinchieri, 2012). PGE_2 is known to possess potent tumor-promoting activity, as shown in animal models and in vitro studies (Nakanishi & Rosenberg, 2013). A study by Kawamori et al. showed that administration of PGE_2 significantly increased the incidence and multiplicity of intestinal adenomas in rats (Kawamori & Wakabayashi, 2002). Studies using $APC^{Min/+}$ mice treated with PGE_2 showed increased epithelial proliferation and Cox-2 expression (Shao et al., 2005).

The specific association between PGE_2 and colorectal cancer is not completely understood. However, it has been demonstrated that PGE_2 receptors indirectly activate Wnt-signaling, PPARγ and epidermal growth factor receptor pathways. Studies have

demonstrated that treatment of colorectal cancer cells with PGE_2 led to the localization of

β-catenin to the nucleus and increased transcription of Wnt target genes such as cmyc,

cyclinD1 etc. Furthermore, it has been hypothesized that the alpha subunit of the PGE_2

receptor may directly inactivate Axin (Castellone et al., 2005). Inactivation of Axin would

lead to loss of function of the APC/GSK-3β/Axin regulatory complex, which is

responsible for the degradation of β-catenin. Loss of the regulatory complex would allow

β-catenin to enter the nucleus and cause cellular proliferation. One possible therapy for

colorectal cancer is direct inhibition of the tumor-enhancing properties of PGE_2.

1.5. The Therapeutic Potential of Non-Steroidal Anti-Inflammatory Drugs

An abundance of clinical research has demonstrated that NSAIDs may possess

therapeutic potential against colorectal cancer. The Cancer Prevention Study II (662,424

patients) and Health Professionals Follow-Up Study (47,900 men), have documented up to

50% reduction in colorectal adenomas, colorectal cancer, and colorectal-associated

mortality in individuals regularly taking NSAIDs (Sandler et al., 2003). Furthermore, it

has been shown that recurrence of adenomas was significantly lower in patients taking

low-dose aspirin (81 mg/day) (Logan et al., 1993). These findings have been confirmed in

animal models of colorectal cancer, where NSAIDs can reduce the number and size of

colorectal carcinoma (Iwama, 2009). In addition to epidemiological studies, Kudo et al.

showed that the NSAID indomethacin inhibited tumour growth in chemically induced

colorectal cancer in rats (1980). Indeed, these results have been demonstrated with other

NSAIDs and genetic models of colorectal cancer (Chan et al., 2005).

Although a solid understanding of the specific mechanism by which NSAIDs have therapeutic potential against cancer is currently lacking, it has been clearly demonstrated that carcinogenesis promotes an inflammatory microenvironment. Furthermore, it has become well established that colon cancer is associated with over-expression of Cox enzymes and accompanying over-production of PGs (Nakanishi & Rosenberg, 2013). It has been shown that several animal and human tumor tissues have high concentrations of PGs (Wang & Dubois, 2006). Several lines of research have illustrated a distinct function of PGE_2 of connecting inflammation to cancer pathogenesis (Cha & DuBois, 2007). In addition to the association of cancer and PGE_2, byproducts of the Cox-2 pathway may cause mutations in DNA and could initiate carcinogenesis (Cha & DuBois, 2007).

NSAIDs have been shown to modulate carcinogenesis by a variety of ways independent of the Cox pathway. Studies have shown NSAIDs interrupt pathways that are commonly over-active in colorectal cancer. One such pathway is nuclear factor kappa B (NF-κB) signaling cascade. NF-kB leads to the transcription of specific genes involved in tumour proliferation, invasion and metastasis (Plummer et al., 1999). Aspirin and other NSAIDs have been shown to inactivate NF-κB in vivo (Li et al., 2011). Another common mutation in colorectal cancer is in p53. Mutations in p53 lead to its inactivation and loss of tumour suppressor activity. NSAIDs have been shown to acetylate p53 and thus increase its ability to bind to DNA and inhibit unregulated replication (Itzkowitz & Yio, 2004). In addition to NSAIDs effect on signaling pathways and proteins, they have been shown to induce apoptosis (programmed cell death), and cell cycle arrest (Itzkowitz & Yio, 2004).

The NSAID-induced gene (NAG-1), stimulated by NSAIDs, is a member of the TGF-β-super family involved in apoptosis and tumourigenesis (Wilson et al., 2003). In

colorectal cancer tissue it has been shown that NAG-1expression is significantly reduced and then unregulated after treatment with NSAIDs (Baek et al., 2006). It has been proposed that NAG-1 contributes to the chemo-preventative actions of NSAIDs (Iguchi et al., 2009).

More recently it has been shown that NSAIDs may have a direct effect on the Wnt pathway. Aspirin, indomethacin, sulindac and celecoxib have been shown to increase phosphorylation and consequential degradation of β-catenin leading to the decrease in cellular proliferation (Brown et al., 2001). Other studies have illustrated that NSAIDs inhibit Wnt/β-catenin transcriptional activity without increasing β-catenin phosphorylation (Stolfi et al., 2013).
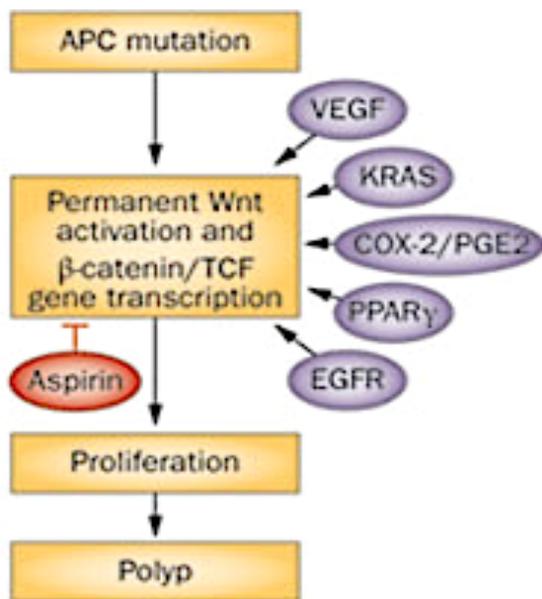
Figure 1.1: The Therapeutic mechanisms of aspirin and NSAIDs. The majority of colorectal cancer is associated with mutations in APC gene leading to the permanent activation of the Wnt pathway β-catenin/TCF gene transcription and consequential cellular proliferation and polyp formation. Both NSAIDs and aspirin inhibit this pathway and the associated increases in Cox-2/PGE2, EGFR, and PPARγ etc. Adapted from (Ulrich et al., 2006)

1.6. Adverse Effects of Conventional NSAIDS

Although the chemo-preventative effects of NSAIDs are promising, there are some adverse effects associated with NSAID use. For instance, NSAID use has been shown to cause adverse effects in the gastrointestinal, renal and cardiovascular systems. Common gastrointestinal adverse side effects of NSAIDs include gastric and intestinal ulceration and bleeding, dyspepsia, diarrhea, and nausea (Bannwarth, 2002). The associated adverse effects of NSAIDs are due to the vital functions PGs perform within the body. PGs are produced by the healthy stomach and small intestine in order to fortify mucosal defense mechanisms and enhance injury repair (Wallace, 2008). Moreover, Cox-1 and Cox-2 are important in maintaining mucosal blood flow, mucus and bicarbonate secretion, angiogenesis, and reducing leukocyte adherence in the upper gastrointestinal tract. It has been shown that NSAIDs can cause direct damage to the gastrointestinal epithelial cells because of their acidic nature. They may cause epithelial cell death by direct contact, can inhibit epithelial cell repair by interfering with epithelial growth factor signaling pathway, and can disrupt the phospholipids on the mucosal surface allowing for acidic damage to the lumen (Lanas et al., 2012). Within the small intestine NSAID associated enteropathy is not only due to the decrease in PG synthesis and direct epithelial damage but is also associated with increase neutrophil infiltration, TNF-α release, and increase in gram-negative bacteria (which in turn induce more epithelial cell damage) (Gargallo & Lanas, 2013). The damage is exacerbated because NSAIDs may undergo entrohepatic recirculation which leads to re-exposure of the epithelium to the NSAIDs (Wallace, 1997).

The renal side effects of NSAIDs include salt and fluid retention, and hypertension. Less frequently, NSAIDs may also cause interstitial nephritis, nephrotic syndrome, acute renal failure and acute tubular necrosis (Cheng & Harris, 2004; Robinson et al., 1990; Simon, 1991). Cardiovascular side effects of NSAIDs include an increase in risk of myocardial infarction and stroke (Roubille et al., 2013).
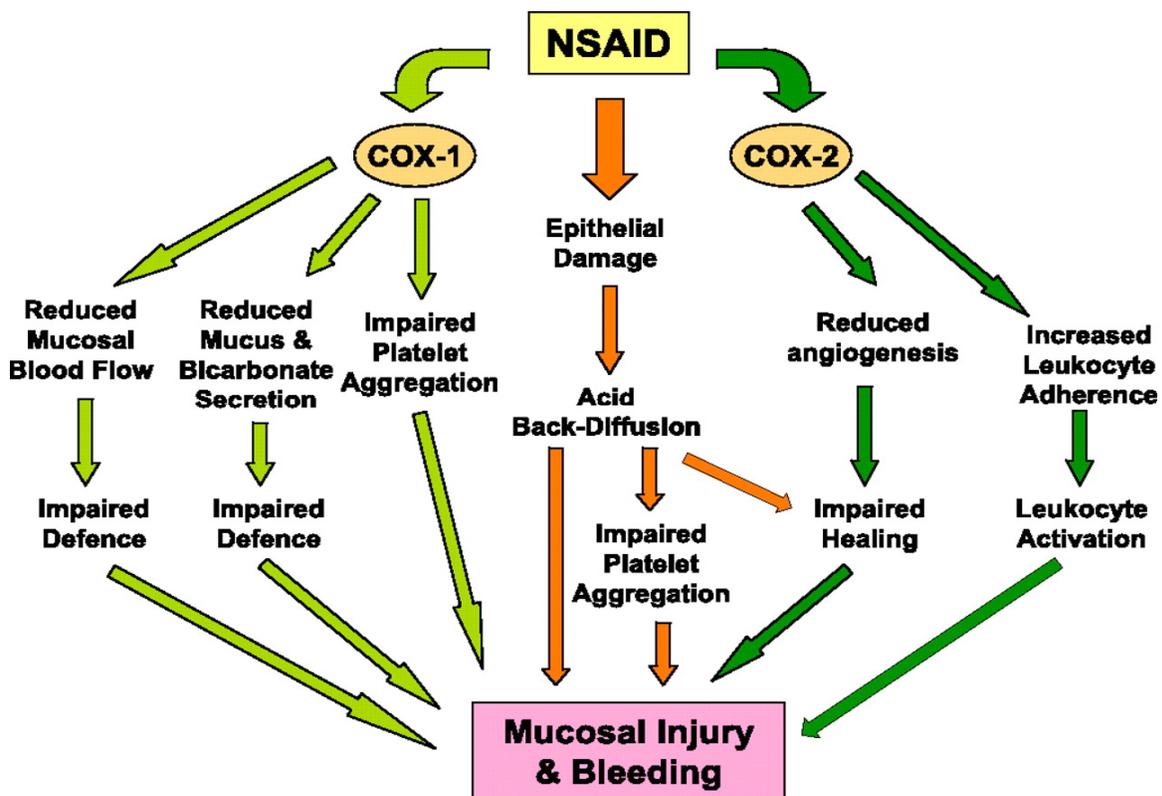
Figure 1.2: Pathogenesis of NSAID-induced gastric injury and bleeding. This figure illustrates the multiple pathways through which NSAIDs may induce mucosal injury and bleeding (Figure from Wallace et al., 2007).

1.7. The Emergence of Hydrogen Sulfide

Hydrogen sulfide (H_2S) is the most recently identified endogenous gaseous mediator. Similar to nitric oxide (NO) and carbon monoxide (CO), H_2S plays a fundamental role in the regulation of cellular and physiologic functions. H_2S is produced throughout the body from L-cysteine. Three distinct pathways of synthesis have been identified. There are two pyridoxal-5'-phosphate (P5P)-dependent pathways; cystathionine-beta-synthase (CBS) and cystathionine-gamma-lyase (CSE). There is also a P5P-independent pathway, which involves the actions of α-ketogluterate-dependent enzyme cysteine-aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3MST) (Szabo, 2007).

H_2S exhibits similar beneficial effects as NO on the cardiovascular system. It acts as an anti-inflammatory agent and demonstrates anti-peroxidase, oxidative stress and angiogenesis activity (Kimura, 2011). H_2S has many functions throughout the body. It functions as an anti-inflammatory mediator, antioxidant, smooth muscle relaxer, and a vasodilator, as well as induces cardio-protective functions, among others. H_2S has been shown to inhibit leukocyte-endothelial cell adhesion and edema. H_2S has also been shown to decrease inflammation by inhibiting NFκB activation and inducing neutrophil apoptosis (Wallace, 2010). Exogenous H_2S donors have also been shown to induce potent anti-inflammatory effects in vivo and in vitro. GYY4137 (a slow releasing H_2S donor) was shown to inhibit the secretion of pro-inflammatory cytokines TNF-α, NO, PGE$_2$ and IL1-β (Li et al., 2008; Whiteman et al., 2010). Furthermore, H_2S acts as an inhibitor of contraction in GI smooth muscle and cardiovascular blood vessels, which leads to vasodilatation. It is also involved in angiogenesis, cardiovascular protection and

modulation of sepsis and, most importantly, it exhibits inhibitory effects on cancer

(Snyder & Paul, 2012).
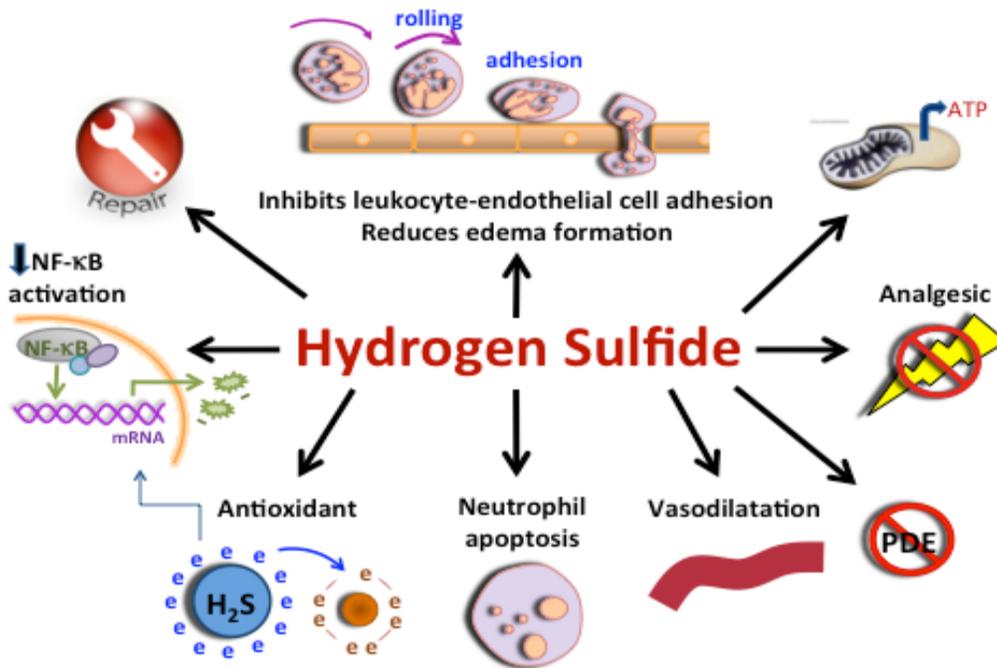
Figure 1.3: The multiple functions of H₂S. This figure illustrates the multiple effects of H₂S within the body (Figure from Wallace, 2012). H2S has been shown to decrease NF-kB activation, perform antioxidant function, induce neutrophil apoptosis, inhibit phosphodiesterase, induce repair of tissues, perform analgesic functions and drive the production of ATP.

Among its plethora of function within the body, H₂S has been demonstrated to act as an antioxidant by activating Nrf2 (Kimura, 2004). Nrf2 is member of the antioxidant response pathway, which is the primary cellular defense pathway against the cytotoxic effects of oxidative stress. Under normal unstressed conditions, Keap1 keeps Nrf2 in the cytoplasm. In the cytoplasm Nrf2 is ubiquitized and is degraded quickly. However, under

oxidative stress Nrf2 is not degraded; rather it enters the nucleus and acts as a transcription factor for antioxidant genes. It binds to antioxidant response elements and initiates their transcription. Activation of Nrf2 results in the induction of many cytoprotective proteins including NAD(P)H quinon oxidoreductase 1 (Nqo1) and p53 binding protein 1 (p53bp1). H_2S has been shown to directly bind to Keap1 and inhibit its proteosomal degradation of Nrf2 (Hourihan et al., 2013).
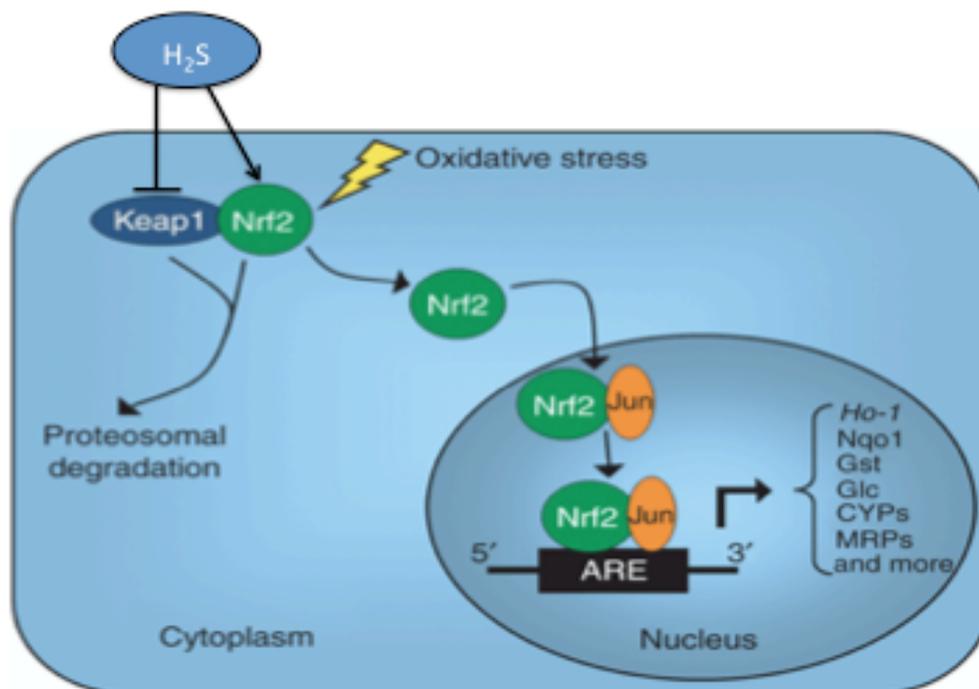
Figure 1.4: The Nrf2 pathway and the activator role H_2S. In the absence of oxidative stress Keap1 binds Nrf2 and causes its proteosomal degradation. In the presence of oxidative stress Keap1 is degraded and Nrf2 translocates into the nucleus where it leads to the transcription of the antioxidant response elements whose proteins perform several functions to protect the cell from oxidative stress. H_2S has been shown to bind to Keap1 and inhibit its association and consequential degradation of Nrf2. (Figure adapted Bataille & Manautou, 2012).

In the gastrointestinal tract H_2S has been shown to be vital in mucosal defense to injury. Furthermore, multiple studies have demonstrated that colonic H_2S synthesis is significantly increased in damage or inflamed tissue (Schicho et al., 2006; Wallace et al., 2009). Suppression of H_2S leads to increases in the basal level of inflammation and susceptibility to ulcerations within the gastrointestinal tract (Wallace, 2010). H_2S donors have been shown to induce resistance of NSAID induced mucosal injury, and accelerate healing of ulcers. These findings have been attributed to the anti-inflammatory and reparative effects of H_2S (Wallace et al., 2007). H_2S may also induce vasodilatation and smooth muscle relaxation, similar to NO and prostaglandins, which is vital for limiting injury and inducing tissue repair post injury (Wallace et al., 2007). H_2S donors have been shown to increase PGE_2 synthesis by increasing Cox-2 expression (Wallace, 2009). PGE_2 as stated above is essential in repair of tissue post injury. H_2S donors have been shown to up regulate anti-inflammatory cytokines such as IL-10 in vivo (Wallace et al., 2009). The ability of H_2S to promote gastrointestinal mucosal defense, healing and resolution makes it essential in inflammatory bowel disease and NSAID-induced gastro-entropathy.

1.8. Hydrogen Sulfide-NSAIDs

The striking quantities of research illustrating H2S's ability to reduce NSAID-induced gastro-entropathy show its value as a therapeutic moiety. A group of NSAIDs, which release hydrogen sulfide, have recently been developed. This new class of NSAIDs combine the analgesic, antipyretic and anti-inflammatory effects of conventional NSAIDs but do not produce the associated gastrointestinal side effects. These H_2S-NSAIDs include; ATB-346 (a H_2S derivative of naproxen) and ATB-352 (a H_2S derivative of

ketoprofen), among others. These two NSAIDs have the conventional NSAID attached to

a 4-hydroxythiobenzamide (TBZ) group, which releases H_2S within the body (Wallace et

al., 2010). These NSAIDs have been evaluated in vivo in healthy rats, rats with

compromised mucosal defense and in rats with models of co-morbidities, such as; aging,

arthritis, obesity, hypertension, etc (Blackler et al., 2012). These H_2S-NSAIDs have been

shown to inhibit effects of inflammation and the cyclooxegenase enzymes but produced

negligible damage in the stomach and intestine compared to their conventional

counterparts. Furthermore, studies of ATB-346 illustrated that it promotes ulcer healing,

does not elevate blood pressure (unlike naproxen) and is a more effective inhibitor of

inflammation (Wallace et al., 2010). These H_2S-NSAIDs provide a promising tool in the

pharmaceutical toolbox. Recently, it has been shown that a number of H_2S–NSAIDs can

be useful in inhibiting colorectal cancer (Chattopadhyay et al., 2012).
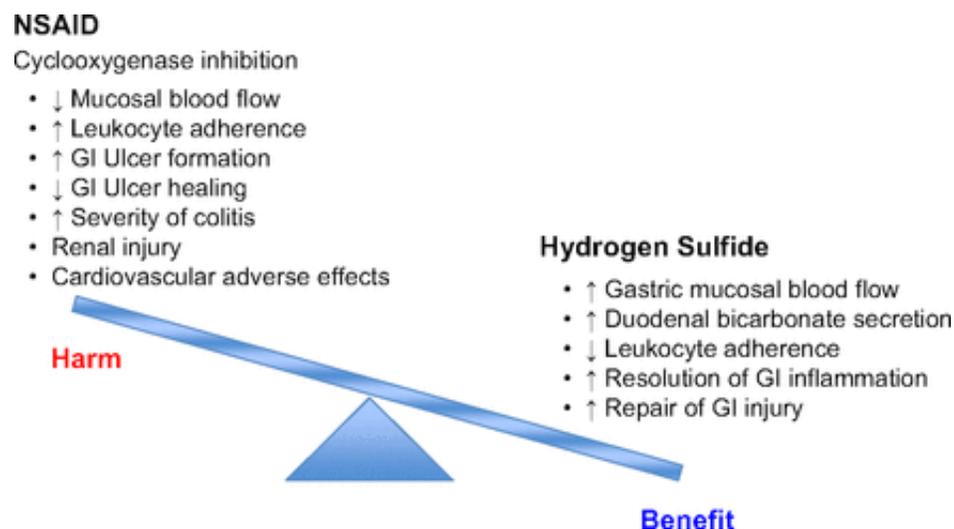
Figure 1.5: H_2S can counteract many of the detrimental effects of NSAIDs on the gastrointestinal tract. The multiple functions of H_2S have been shown to restore the adverse effects associated with NSAID use (Figure from Chan & Wallace, 2013).

1.9. Hydrogen Sulfide and Colorectal Cancer

Recent publications have shown that H_2S levels are significantly higher in patients who have previously undergone resections for sigmoid cancer and later developed metachronous lesions. Rhodanese (RHOD) and thiol methyltransferase (TMT), the two H_2S-detoxifying enzymes in the human colon, are markedly reduced in advanced colon cancer leading to increased research into the effects of H_2S in colorectal cancer (Ramasamy et al., 2006). In addition, inconclusive in vitro studies illustrate that H_2S may increase apoptosis, induce cell cycle arrest, reduce growth ability and suppress migration in colorectal cancer cell lines. Several publications have demonstrated that H_2S donors can kill human gastric cancer cells *in vitro* and are cytotoxic towards human breast cancer cells. Furthermore, H_2S donors inhibit cell proliferation by causing gastric cancer cell arrest in the M-phase. These studies have found that H_2S donors my cause cell death, primarily by apoptosis. These cells were found to have down-regulation of Bcl-2 (an anti-apoptotic protein) and increased caspase-3 (a pro-apoptotic protein) expression and activity (Deplancke & Gaskins, 2003; Kashfi, 2013; Ma et al., 2011; Wu et al., 2012). Other potential targets of H_2S on apoptosis include p38 and caspase-3, MEK and JNK, and heat shock protein (HSP-90) (Yang et al., 2006).

Several H_2S donors have been studied in vitro for their ability to inhibit cancer development. A study by Lee et al. demonstrated that NaHS and GYY4137, two H_2S donors one a fast releaser of H_2S and one slow releaser of H_2S, respectively, did not affect the survival of normal lung fibroblasts but GYY4137 promoted cancer cell apoptosis and induced Gap-2/Mitotic-phase cell cycle arrest (Lee et al., 2011a).

Diallyl disulfide (DADS) is an organosulfur compound found in garlic and releases hydrogen sulfide within the body; it has been shown to exhibit anti-cancer effects. It has been shown to decrease carcinogen induced cancers and inhibit the proliferation of various types of cancer cells (Fukushima et al., 1997). Filomeni et al. have shown that DADS may induce cell death in neuroblastoma, prostate cancer, gastric cancer, breast cancer, lung adenocarcinoma, and colon cancer lines (Filomeni et al., 2003). DADS has been shown to induce apoptosis and induce cell cycle arrest at Mitotic-phase in human gastric cancer cell lines (Das et al., 2007). DADS was shown to induce apoptosis through down regulation of Bcl-2 and increased expression and activity of caspase-3 (Mice et al., 2012).

1.10. Objectives

Chemo-preventatives for colorectal cancer are a current area of increased interest. Several clinical trials have already begun using NSAIDs with various forms of cancer. The most limiting factor of NSAIDs is their associated adverse effect. H_2S-NSAIDs are a class of NSAID that have been shown to produce equivalent anti-inflammatory effect to conventional NSAIDs without inducing the associated gastro-entropathy. Furthermore, studies have illustrated that H_2S induces anti-inflammatory, reparative and anti-tumourgenic functions that may hold value in preventing colorectal cancer. The first section of this thesis will examine the effect of the azoxymethane induced ACF model of colorectal cancer on endogenous H_2S and then address the chemo-preventative effects of ATB-346 and ATB-352 in this model. The second section of this thesis will focus on the chemo-preventative effects of ATB-346 and ATB-352 in an APC$^{Min/+}$ mouse model of

colorectal cancer and address possible mechanism of action. This thesis will cover the following objectives:

1. To compare colonic H_2S production in both healthy and azoxymethane-treated mice.

2. To compare the chemo-preventative effects of ATB-346 and naproxen in an azoxymethane-induced ACF and APC$^{Min/+}$ model of colorectal cancer.

3. To compare the chemo-preventative effects of ATB-352 and ketoprofen in an azoxymethane-induced ACF and APC$^{Min/+}$ model of colorectal cancer.

4. To examine the contribution of TBZ (the H_2S releasing moiety of ATB-346 and ATB-352) in the chemo-preventative effects of the ATB-346 and ATB-352.

5. To explore the possible mechanism of the chemo-preventative effects of ATB-346.

6. To explore the chemo-preventative effects of diallyl disulfide in an APC$^{Min/+}$ model of colorectal cancer.

2.0 GENERAL MATERIALS AND METHODS

Many methods and materials were employed throughout these studies. Materials and methods pertinent to a specific study are detailed in appropriate chapters.

2.1. Animals

Male A/J, APC$^{Min/+}$ and C57BL/6 were purchased from Jackson Laboratories (Bar Harbor, MA, USA) and housed in the Central Animal Facility at McMaster University. The mice were fed standard chow and water *ad libitum*, and were housed in a room with controlled temperature ($22 \pm 1°C$), humidity (65–70%) and light cycle (12 hours of light/12 hours of dark). The Animal Care Committee of the Faculty of Health Sciences at McMaster University approved all experimental procedures. The studies were carried out in accordance with the guidelines of the Canadian Council of Animal Care. The health of the animals was assessed at least twice per day, and any animals in distress or having lost >15% of their original body weights were euthanized by an overdose of sodium pentobarbital.

2.2. Measurement of Hydrogen Sulfide Production

To determine the colons' ability to produce H_2S, a modified version of the Stipanuk and Beck (1982) assay was used to measure H_2S (Stipanuk & Beck, 1982). In this procedure sulfide was trapped by zinc acetate, which was subsequently acidified with NNDP and $FeCl_3$ to produce methylene blue. Colour intensity of methylene blue was measured using a spectrophotometer, which was used to measure the relative levels of

H$_2$S. Colonic tissue was homogenized in ice-cold 50mM potassium phosphate buffer (pH 8.0; 12% w/v), with a Polytron homogenizer (Kinematic, Bohemia, NY, USA). Tissue homogenates (0.5 mL), buffer (0.4 mL) and substrates of H$_2$S reaction were added to large scintillation vials resulting in a final volume of 1 mL. A 2-mL tube containing a piece of filter paper (0.5 by 1.5 cm) soaked in zinc acetate (1%; 0.3 mL) was placed inside of the larger scintillation vial. The scintillation vials were then flushed with nitrogen gas for 20 seconds and capped with an airtight serum cap. The vials were then transferred to a 37°C shaking water bath for 90 minutes. Vials were then transferred to wet ice for 10 minutes, after which trichloroacetic acid (TCA; 50%; 0.4 mL) was injected into the reaction mixture through the serum vial cap. Thereafter the vials were transferred to a 50°C shaking water bath for 60 minutes to allow for the trapping of evolved H$_2$S by the zinc acetate. The zinc acetate used to trap H$_2$S did not come into contact with homogenized tissue. The serum cap was then removed and NNDP (20 mmol; 50 μL) in 7.2 M HCl and FeCl$_3$ (30 mmol/L; 50 μL) in 1.2 M HCl were added to the inner tube containing zinc acetate. Samples were left to stand in the dark for 20 minutes. Absorbance at 670 nm was measured with a micro-plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). A calibration curve of absorbance versus H$_2$S concentration was generated using NaHS of varying concentrations.

2.3. Immunoassay of Prostaglandin E$_2$

Mouse colonic samples were excised, weighed and minced for 30 seconds in a 1 mL solution of sodium phosphate buffer (10 mmol/L; pH 7.4). The tube containing the minced sample and buffer were then left in a shaking water bath (37°C) for 20 minutes.

Samples were then removed and centrifuged for 30 seconds at 14, 000 g. The supernatant was collected and stored at -80°C. Using ELISA (Caymn Chemical Company, Ann Arbor, MI, USA), PGE_2 concentration was determined from the collected supernatant.

2.4. Measurement of Myeloperoxidase Activity

Myeloperoxidase (MPO) is an enzyme found predominantly in granules of polymorphonuclear leukocytes and has been used as an indicator of tissue inflammation. To measure MPO activity frozen tissue was thawed and suspended in 0.5% hexadecyltrimethylammonium bromide (pH 6.0; 50 mg of tissue per milliliter) and subsequently homogenized for 15 s using a Polytron homogenizer (Kinematic, Bohemia, NY, USA). The homogenates were then centrifuged for 2 minutes at 14, 000 g. Following centrifugation, the supernatants from each sample were collected and added to a mixture of potassium phosphate buffer, distilled H_2O, O-dianisidine dihydrochride and 1% hydrogen peroxide. MPO activity was measured using spectro-photometric assay. A change in absorbance was measured at a wavelength of 460 nm over 30 second intervals (spectraMax M3, Molecular Devices Corporation, Sunnyvale, CA, USA) and converted to a Kmax value using SoftMax Pro 5.4 software. These values were then converted to average units of MPO activity per mg of tissue.

2.5. Western Blot Analysis

Western blot analysis was used to determine expression of several proteins. Mouse tissue was homogenized in ceramic bead tubes (1.8 mm tubes) (Mo Bio Labrotory Inc., Carlsbad, CA, USA) using Precellys 24 homogenizer (Bertin Technologies Corporations,

Paris, France) for 3 intervals of 35 seconds at 6500 rpm. In a lysis buffer containing

50 mmol/L Tris (pH 8.0), 0.5% NP-40, 1 mmol/L EDTA, 150 mmol/L NaCl, 10%

glycerol, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 1 mmol/L

sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and a tablet of protease

inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Proteins were separated on

4–20% gradient polyacrylamide gels (Bio-Rad, Canada). Blots were incubated with

blocking buffer (5% Bovine Serum Albumin Tris-buffered saline and Tween 0.05%) for 1

hour (Sigma, St. Louis, MO). They were then incubated in primary antibody overnight at

4°C. The blots were then washed with Tri-Buffered Saline containing 0.05% tween (3 x 10

min). The blots were incubated (1 hour, room temperature) with secondary anti-rabbit IgG

antibody conjugated to horseradish peroxidase (1:1000). Enzymes were visualized using

an enhanced chemiluminescence detection kit on a Chemi-doc gel imaging system (Bio-

Rad, Canada). The intensity of the bands was determined and analyzed using ImageLab

2.0 software (Bio-Rad, Canada). The expression of each enzyme was normalized to the

expression of β-actin (Cell Signaling Technology, Beverly, MA, USA, 1:1000).

2.6. Quantitative Polymerase Chain Reaction

Total RNA was extracted from tissues using a QIAGEN RNeasy Mini Kit (Qiagen,

Germany). The concentration and purity of the total RNA was estimated using a

NanoDrop® ND-1000 UV-Vis spectrophotometer by measuring the absorbance at 260 and

280 nm. Complementary DNA (cDNA) was synthesized from 0.3 µg total RNA for each

sample using a QIAGEN QuantiTect Reverse Transcription Kit. Real-time PCR was

conducted with QuantiTect SYBR Green PCR Kit on a Rotor Gene 3000 Cycler (Corbett,

Australia). The cycling conditions of 40 cycles were 94°C/15 s, 55°C/30 s and 72°C/30 s. Each sample was run in triplet repeat and normalized by β-actin. For each PCR run, a non-template reaction was included as negative controls.

2.7. Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM). Comparisons among groups of data were performed by one-way analysis of variance followed by a post hoc test (Dunnett's Multiple Comparison Test for parametric data and Mann Whitney Test for non-parametric data). An associated probability (p value) of less than 5% was considered significant.

2.8. Test Drugs and Materials

ATB-346 (2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester) and ATB-352 were provided by Antibe Therapeutics Inc. (Toronto, ON, Canada). Azoxymethane, naproxen, ketoprofen, L-cysteine, P-5-P, $FeCl_3$, N,N'-dimethyl-p-phenylenediamine sulfate salt, sulfathiozole, methylene blue, zinc acetate, and NaHS were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isoflurane was obtained from Abbott Laboratories (Montreal, Canada). TBZ (4-hydroxythiobenzamide) was purchased from SynChem Inc. (Des Plaines, IL, USA). ELISA kits for measuring PGE_2 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Isoflurane was obtained from Abbott Laboratories (Montreal, Quebec, Canada).

3.0 CHEMOPREVENTATIVE EFFECTS OF NAPROXEN AND HYDROGEN SULFIDE RELEASING NAPROXEN DERIVATIVE (ATB-346) IN AZOXYMETHANE INDUCED ABARRENT CRYPT FOCI.

3.1.   Introduction

In this study we examined the chemo-preventative effects of ATB-346 (a H_2S-derivative of naproxen) and ATB-352 (a H_2S derivative of ketoprofen), and their parent compounds in an azoxymethane-induced ACF model of colorectal cancer. We found that ATB-346 had superior ability to inhibit ACF formation compared to naproxen. These findings were unique to ATB-346 as ATB-352 or TBZ (the hydrogen sulfide releasing group on the derivatives) displayed effects similar to the parent drugs ketoprofen and naproxen, respectively. We also studied the effects that ACF development had on colonic H_2S synthesis and the expression of H_2S-producing enzymes. We determined that this model of colorectal cancer did not lead to changes in colonic H_2S synthesis or gene expression of H_2S-producing enzymes. Overall, these findings suggest that the H_2S-releasing NSAID ATB-346 represent a safer, more efficacious chemo-preventative treatment than traditional NSAIDs.

3.2.   Materials and Methods

Animals

Male A/J, mice (20 to 25 g) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). They were fed standard laboratory rodent chow, tap water *ad libitum*, and were housed in a room with controlled temperature (22+/- 1°C), humidity (65-70%) and

light cycle (12 hours light/ 12 hours dark). The mice were monitored at least twice daily

and weighed weekly. All studies were approved by the Animal Care Committee of the

Faculty of Health Sciences at McMaster University and performed in accordance with the

guidelines of the Canadian Council of Animal Care.

Induction and quantification of aberrant crypt foci

Five-week old mice were given injections of azoxymethane (10 mg/kg, i.p.) at

weekly intervals (total of 4 injections). The mice were euthanized one week after the final

injection of azoxymethane. A laparotomy was performed and the entire colon was

excised. After gentle flushing with 0.9% saline, the colon was tied at both ends with silk

sutures and insufflated with 10% phosphate-buffered formalin. The colons were

submerged in formalin for 24 hours then stained with 0.2% methylene blue. Using a

dissection microscope at 40x magnification, the number of ACF in the entire colon was

blindly determined. ACFs are pre-cancerous tumours, which were distinguishable from the

surrounding normal crypts by their increased size, significantly increased distance from

the lamina to basal surface and easily discernible pericryptal zone (Ward et al., 1973).

ACF quantification method was performed according to previously published criteria

(McLellan & Bird, 1988).

Naproxen and ATB-346 Administration

During the first two weeks of azoxymethane treatment mice were treated orally

each day with equimolar doses of naproxen, ATB-346 or TBZ. Control mice were treated

with an equivalent volume of the vehicle (95:5, 1% CMC:DMSO).

Ketoprofen and ATB-352 Administration

During the first two weeks of azoxymethane treatment mice were treated orally each day with ketoprofen or ATB-352 (at 130 μmol/kg). Control mice were treated with an equivalent volume of the vehicle (95:5, 1% CMC:DMSO).

Measurement of Prostaglandin Levels

Colonic PGE_2 levels were measured in mice treated with naproxen, ATB-346 or vehicle (at 130 μmol/kg), as described previously (Wallace, 2000). Briefly, samples of the colon were excised, weighed, and added to a tube containing 1 mL of sodium phosphate buffer (10 mmol/L; pH 7.4). The tissue sample was minced with scissors for 30 seconds then placed in a shaking water bath (37°C) for 20 minutes. The samples were centrifuged (9000 g) for 1 minute, and the concentrations of PGE_2 in the supernatants were determined by ELISA.

Myeloperoxidase activity

Colonic inflammation in the above-mentioned mice was assessed by the measurement of myeloperoxidase (MPO) activity, as previously described (Wallace et al., 1998). MPO has been used extensively as a quantitative index of granulocyte infiltration.

Measurement of H_2S synthesis

The ability of colonic tissue to produce H_2S was measured using a previously described zinc trapping, methylene blue assay (Stipanuk and Beck, 1982), with

modifications (Wallace et al., 2007). In this assay H_2S synthesis was measured from tissue homogenates incubated in the presence of the substrate L-cysteine and different cofactors required by H_2S producing pathways. H_2S production was measured via the CSE/CBS pathways or the CAT-3MST pathways as previously described (Flannigan et al., 2013). H_2S synthesis via CSE and CBS required the presence of P5P (2 mM), while that via CAT-3MST required α-KG (0.1 mM).

mRNA Expression of H₂S producing enzymes

Snap frozen azoxymethane or vehicle-treated colonic samples were used to detect changes in gene expression of H_2S-producing enzyme. Briefly, RNA was extracted from samples using a RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Two-step, real-time, reverse transcription polymerase chain reaction was performed as described (QuantiTect Reverse Transcription kit) to generate template cDNA. 50 ng of template cDNA was used for each reaction with QuantiTect SYBR Green master mix. Validated primer sets for mouse 3MST, CAT, CSE, CBS and β-actin were used (Qiagen). All data were recorded and analyzed using Realplex software (Eppendorf). Target genes were normalized against β-actin.

Reagents

ATB-346 (2-(6-methoxy-napthalen-2-yl)-propionic acid 4-thiocarbamoyl-phenyl ester) and ATB-352 were provided by Antibe Therapeutics Inc. (Toronto, ON, Canada). Azoxymethane, naproxen, ketoprofen, L-cysteine, P-5-*P*, $FeCl_3$, *N,N′*-dimethyl-*p*-phenylenediamine sulfate salt, sulfathiozole, methylene blue, zinc acetate, and NaHS were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isoflurane was obtained from Abbott

Laboratories (Montreal, Canada). TBZ was purchased from SynChem Inc. (Des Plaines, IL, USA). ELISA kits for measuring PGE_2were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

Statistical Analysis

All data are expressed as the mean ± SEM. Comparisons among groups of data were performed by one-way analysis of variance followed by a post hoc test (Dunnett's Multiple Comparison Test for parametric data and Mann Whitney Test for non-parametric data). An associated probability (p value) of less than 5% was considered significant.

3.3. Results

Azoxymethane induced extensive aberrant crypt foci formation in the colon

Treatment with azoxymethane led to pronounced number of ACF in the colon and no macroscopically visible tumors were observed in any of the mice. ACF were observed from the cecum to the sigmoid colon with the above-described characteristics of ACF. ACF were found throughout the colon equally with no particular area of concentration. Across the various studies, a range in number of ACF (40 to 55) was observed in the colons of the mice.

ATB-346 treatment led to superior chemoprevention than naproxen

Mice in all groups tested appeared healthy throughout the experiment with no apparent signs of toxicity. Naproxen treatment demonstrated modest reduction in the number of ACF. Of the doses tested only the two highest doses of naproxen led to a

significant reduction in the number of ACF observed. At the two highest doses of 43 and 130 μmol/kg naproxen significantly reduced the number of ACF by 41 ±4% and 31 ±2%, respectively (*p<0.05) (Figure 3.1). The two lower doses (4.3 and 13 μmol/kg) had no effect on the number of ACF observed.

Unlike naproxen treatment, a significant reduction in the number of ACF was observed in all ATB-346 treatment groups when compared to the vehicle-treated group. The highest dose of ATB-346 tested (130 μmol/kg) showed the strongest inhibitory effect and decreased the total number of ACF by 77 ±2% as compared with the control group (*p<0.05) (Figure 3.1). ATB-346 treatment at 4.3, 13 and 43 μmol/kg led to a 31 ±4%, 43 ±3% and 62 ± 2% reduction in number of ACF compared to the control group, respectively (*p<0.05) (Figure 3.1). Mice administered ATB-346 developed significantly fewer ACF compared to their naproxen dosage counterparts (*p<0.01) (Figure 3.1). These results suggest that ATB-346 exerts a protect effect that was not observed with naproxen treatment.
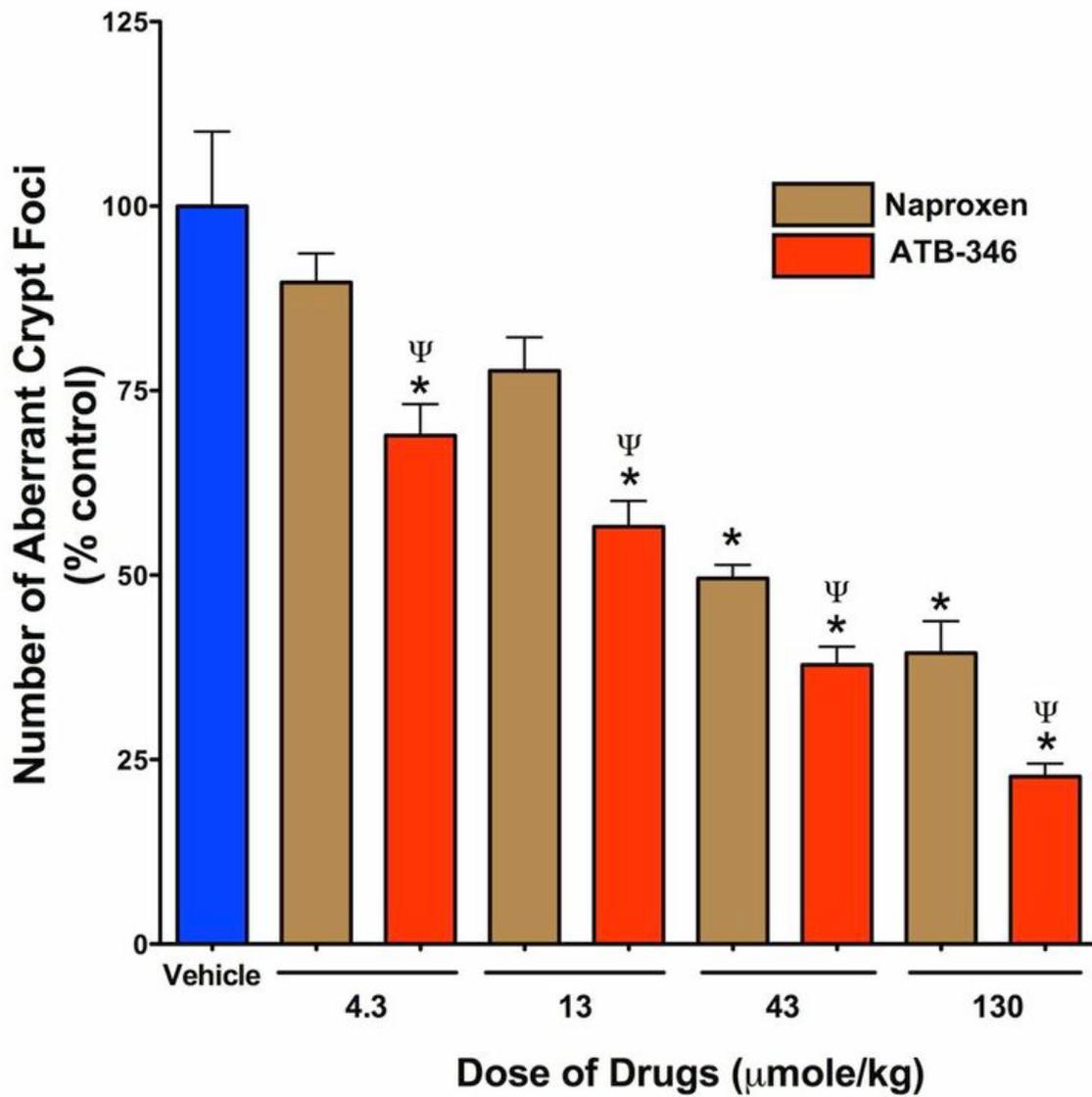
Figure 3.1: Suppression of azoxymethane induced ACF formation in mice colon by

ATB-346, naproxen or vehicle. *P<0.05 ATB-346-treated group vs. vehicle-treated

group, naproxen treated group vs. vehicle. ΨP<0.05 ATB-346-treated group vs. naproxen-

treated group. Data are expressed as mean ± S.E.M. n of 6 per group.

ATB-346 and naproxen decreased colonic PGE$_2$

To determine whether treatment with ATB-346 and naproxen reduced inflammation we measured colonic levels of inflammation in mice with colorectal cancer treated with ATB-346 or naproxen. Mice treated with ATB-346 or naproxen (130 µmol/kg) had a significant reduction in PGE_2 levels compared to vehicle-treated mice (Figure 3.2).
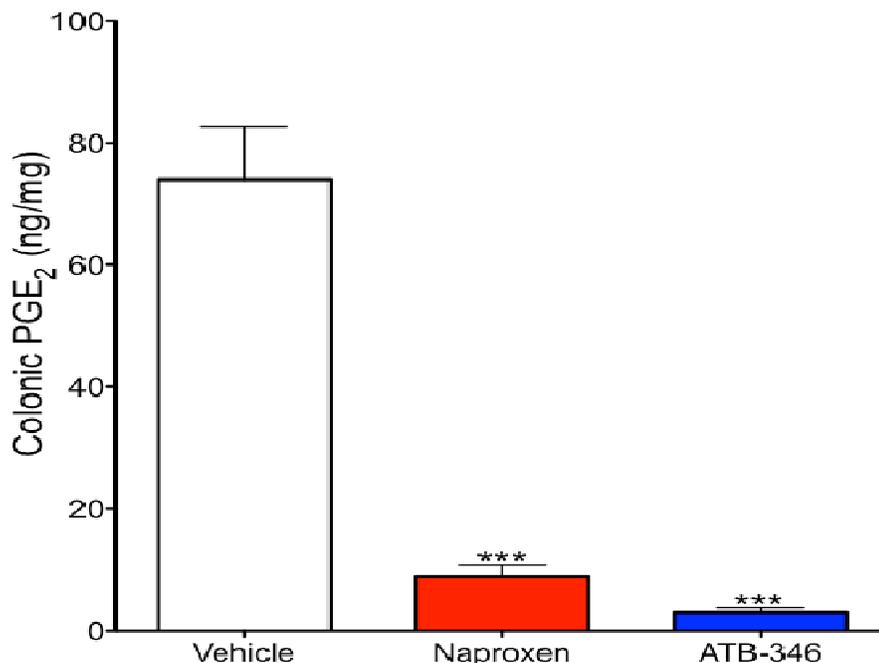
Figure 3.2: Reduction of colonic PGE$_2$ levels by ATB-346 and naproxen (103 µmol/kg) ***p<0.001 naproxen-treated group vs. vehicle-treated group, ATB-346-treated group vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 6 per group

TBZ treatment did not have an effect on number of ACF

Mice were treated with TBZ, the hydrogen sulfide releasing group in ATB-346 and ATB-352 at doses of 4.3, 13, 43, 130 µmol/kg. The results indicate that TBZ at all doses

tested caused no significant reduction in the number of ACF in the colon compared to vehicle-treated mice (Figure 3.3).
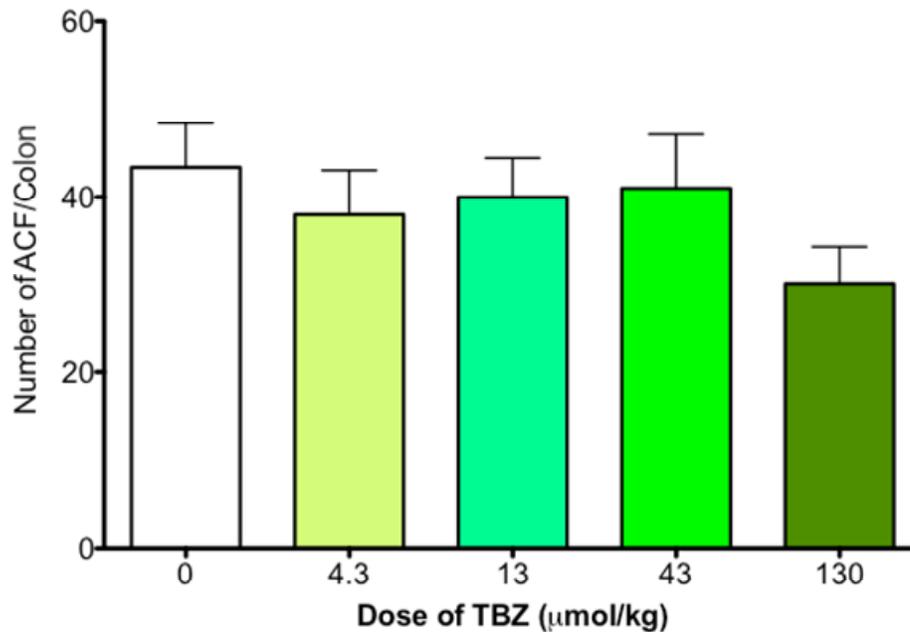
Figure 3.3: Suppression of azoxymethane induced ACF formation in mice colon by TBZ. Data are expressed as mean ± S.E.M. n of 6 per group.

ATB-352 and ketoprofen treatment reduced the number of ACF

A significant reduction in the number of ACF in mice treated with ATB-352 or ketoprofen (130 µmol/kg) was observed when compared to control mice. Ketoprofen treatment led to a 40 ±4% reduction in the number of ACF observed compared to vehicle-treated mice ($p<0.0001$). ATB-352 treatment led to a 50±5% decrease in the number of ACF observed compared to vehicle-treated mice ($p<0.0001$) (Figure 3.4).
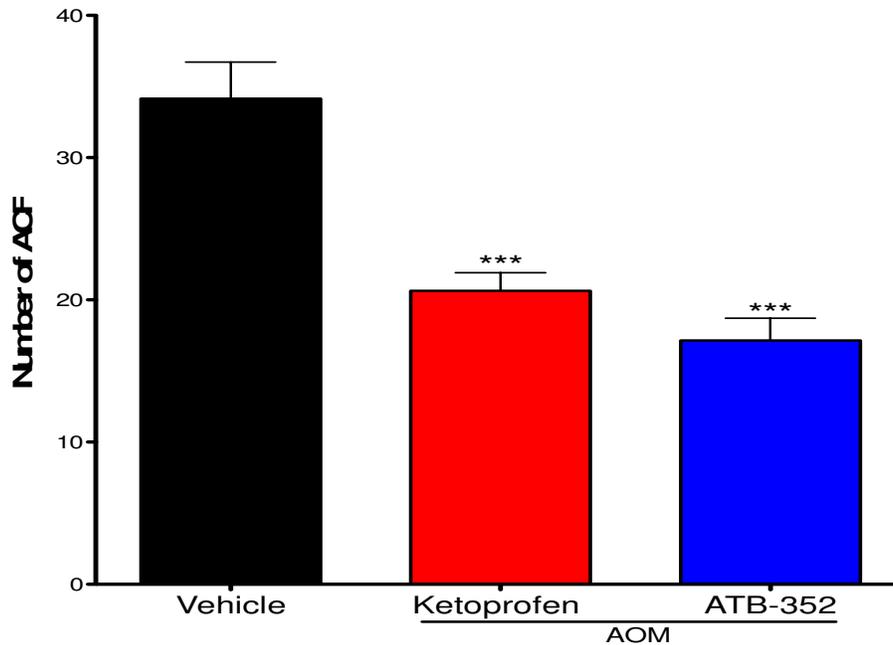
Figure 3.4: Suppression of Azoxymethane induced ACF formation in mice colon by ketoprofen, ATB-352 (103 μmol/kg) or vehicle. ***p<0.001 ketoprofen-treated group vs. vehicle-treated group, ATB-352-treated group vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 8 per group.

Azoxymethane treatment increased colonic MPO levels but caused no change in H_2S synthesis or mRNA expression

Induction of ACF by azoxymethane caused no significant change in the mRNA levels of the H_2S enzymes (Figure 3.5). Colonic CSE, CAT, 3MST, and CBS mRNA levels of mice treated with AOM were similar to saline-treated mice (Figure 3.6). Furthermore, mice treated with azoxymethane had no change in colonic H_2S synthesis compared with saline-treated mice (Figure 3.7). In contrast, mice treated with azoxymethane had a significant increase in colonic MPO activity (p<0.001) (Figure 3.7).
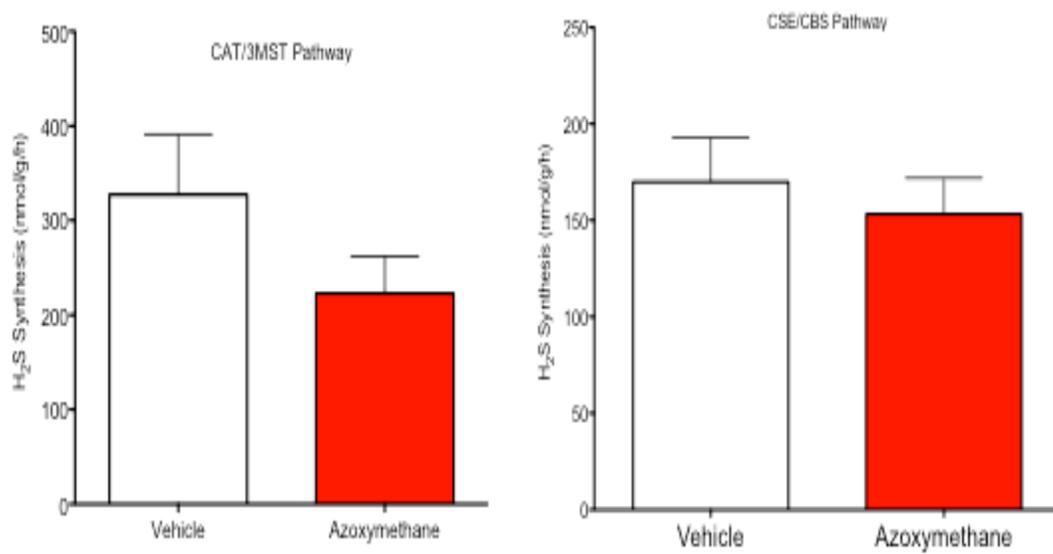
Figure 3.5: The effect of azoxymethane treatment on H$_2$S synthesis. Azoxymethane-treated mice did not have a significant change in both pathways of H$_2$S pathways. Data are expressed as mean ± S.E.M. n of 6 mice per group.
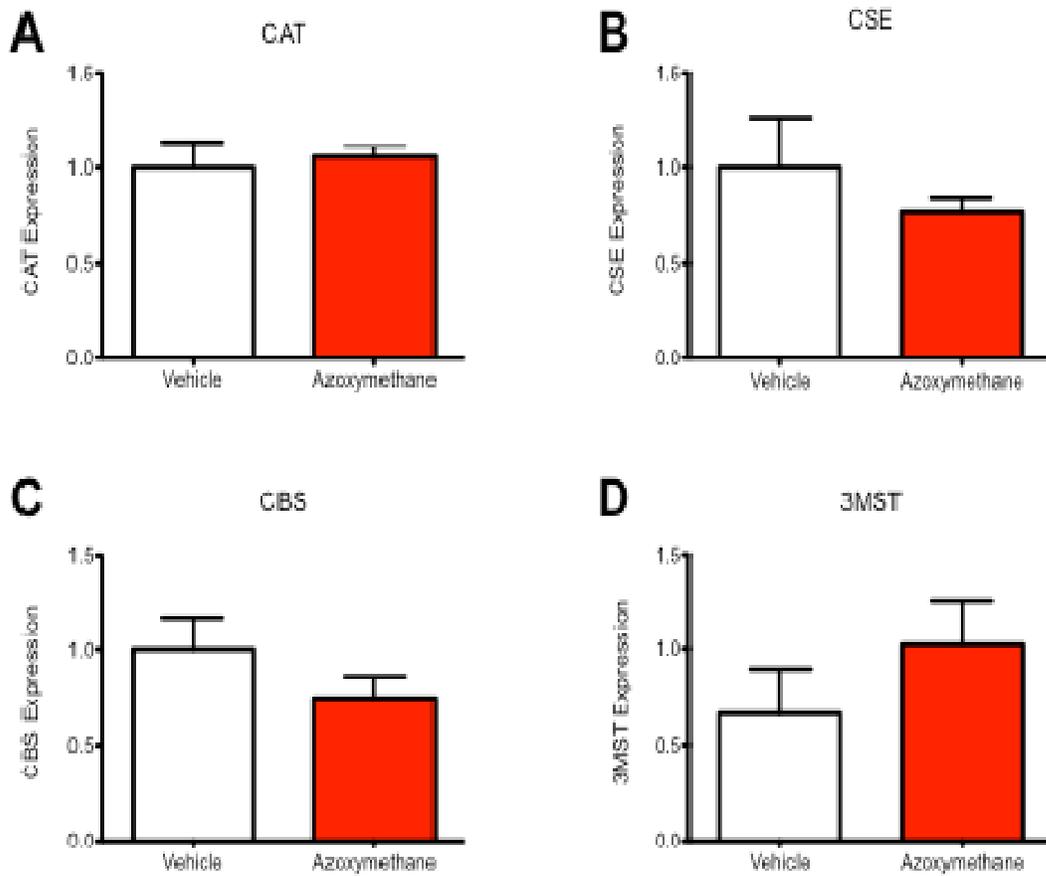
Figure 3.6: The effect of azoxymethane treatment on A) CAT, B) CSE, C) CBS, D) 3MST mRNA expression. The mRNA expression levels were determined by RT-PCR analysis. Data are expressed as mean ± S.E.M. n of 6 mice per group.
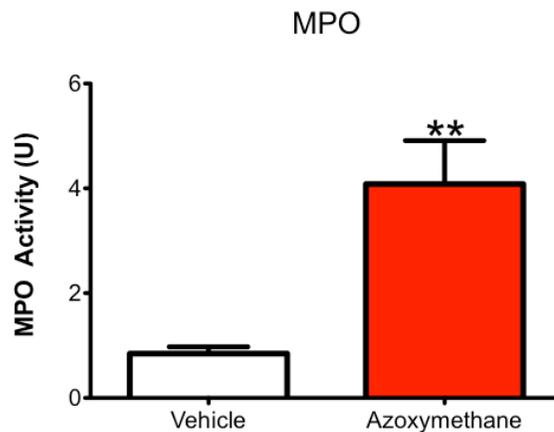
Figure 3.7: The effect of azoxymethane treatment on colonic MPO activity. Data are expressed as mean ± S.E.M. n of 6 mice per group.

3.4. Conclusion

Our results clearly indicate that ATB-346 exhibits superior chemo-preventative effects to its conventional counterpart, naproxen. Using the azoxymethane induced ACF model of colorectal cancer we determined that ATB-346 reduced formation of ACF at all doses tested. At the lowest doses tested we found that ATB-346 inhibited ACF formation, however naproxen at low doses did not reduce incidence of ACF. To determine if the superior chemo-prevention was associated with the hydrogen sulfide group of the drug, we tested TBZ, the hydrogen sulfide moiety in ATB-346 and ATB-352. We found TBZ at all doses did not change ACF formation. Both ATB-346 and naproxen both significantly inhibited PGE_2. In contrast to the superior effects observed with ATB-346, ATB-352 produced equivalent chemo-preventative effect to its conventional counterpart ketoprofen.

We determined that this model of colorectal cancer did not lead to changes in colonic H_2S synthesis or gene expression of H_2S-producing enzymes.

In summary, our findings suggest that the H_2S-releasing NSAID ATB-346 represents a safer, more efficacious chemo-preventative treatment than traditional NSAIDs. These findings may have important implications in the prevention of colorectal cancer in susceptible patients.

4.0 CHEMOPREVATIVE EFFECTS OF HYDROGEN SULFIDE NAPROXEN AND KETOPROFEN DERATIVES (ATB-346 AND ATB-352) IN APC^{MIN/+} MICE MODEL OF COLORECTAL CANCER.

4.1.   Introduction

We have previously demonstrated that ATB-346 was able to significantly reduce the number of ACF induced by the colon carcinogen azoxymethane. In this experiment we have found that ATB-346 may completely inhibit the formation of polyps and tumours in the small intestine and colon in an APC$^{Min/+}$ mice. In the present study, we demonstrated that ATB-346 was a potent inhibitor of polyp/tumour formation in APC$^{Min/+}$ mice. We have illustrated an optimal dose, route and length of treatment for the associated effects. ATB-346 induced a reduction in cellular levels of β-catenin and cmyc without significantly altering levels of APC, or p53.

We tested ATB-346 at different length of treatment, using different routes of administration, and the therapeutic potential of ATB-346. Furthermore, we tested DADS an H$_2$S donor that does not use TBZ. This study also tested the chemo-preventative effects of ATB-352 and its conventional counterpart ketoprofen.

4.2.   Materials and Methods

Animals

Male C57BL/6 and C57BL/6- Apc$^{Min/+}$ (Apc$^{Min/+}$) mice were from Jackson Laboratories (Bar Harbor, MA, USA). All mice were housed in the Central Animal Facility at McMaster University. The mice were fed standard chow and water *ad libitum*, and were housed in a room with controlled temperature (22 ± 1°C), humidity (65–70%)

and light cycle (12 h light/12 h dark). The Animal Care Committee of the Faculty of

Health Sciences at McMaster University approved all experimental procedures. The

studies were carried out in accordance with the guidelines of the Canadian Council of

Animal Care. The health of the animals was assessed at least twice per day, and any

animals in distress or having lost >15% of their original body weight were euthanized by

an overdose of sodium pentobarbital.

Quantification of polyp/tumour score in Apc$^{Min/+}$ mouse model of colorectal cancer

Mice were euthanized, by isoflurane, and the intestines and colons were removed.

The size and number of polyps/tumours were scored blindly. Score of polyps/tumours was

calculated as the size of a polyp/tumour multiplied by the number of polyps/tumours.

After intestines and colons were scored, sections containing polyps/tumours were removed

from each mouse, snap frozen and stored at -80 degrees Celsius. The number and size

(diameter) of polyps and tumors were scored blindly under a dissecting microscope as

described previously (Baek et al., 2006; Sukhthankar et al., 2008).

Determination of macroscopic appearance of polyps/tumours

To determine at what age Apc$^{Min/+}$ mice develop macroscopically visible

polyps/tumours we euthanized mice at 5, 6, 8, 10, 12 and 14 weeks of age. After

euthanization we analyzed the mice for appearance of polyps/tumours.

Treatment protocol with ATB-346, naproxen, and vehicle

6-week-old Apc$^{Min/+}$ mice were treated daily for 14 days with naproxen (10 mg/kg),

ATB-346 (14.5 mg/kg eqimolar to 10 mg/kg of naproxen) or vehicle (95:5, 1%

CMC:DMSO) (orally). Mice were then left until 14 weeks of age. At 14 weeks of age the mice were euthanized with isoflurane. Any anatomical abnormalities were recorded. The small intestine and colon were excised, opened longitudinally and the number and size (in mm) of polyps/tumours were determined by a blinded observer. The size and number of polyps/tumours was used to calculate a total polyp score. After intestinal and colonic scoring sections containing polyps/tumours were removed from each mouse, they were snap frozen and stored at -80 C.

Treatment protocol with ATB-352, ketoprofen, TBZ and vehicle

To determine if the chemo-preventative effects of ATB-346 are unique to ATB-346, we tested whether ATB-352, which is a H_2S releasing derivative of ketoprofen, and TBZ, the hydrogen sulfide releasing moiety in ATB-346 and ATB-352, have similar chemo-preventative effects. We tested the effects of ketoprofen (10 mg/kg p.o.), ATB-352 (15.2 mg/kg equimolar to ketoprofen dose, p.o.) and TBZ (4.5 mg/kg equimolar to ketroprofen dose, p.o.) on an $APC^{Min/+}$ model of colorectal cancer. 6-week-old mice were treated with ketoprofen, ATB-352, TBZ or vehicle for 14 days. At 14 weeks of age the mice were euthanized and polyp score was determined.

Determining the optimal duration of treatment with ATB-346

To determine the optimal length of treatment with ATB-346, $Apc^{Min/+}$ mice were treated daily with ATB-346 at 14.5 mg/kg for 3, 7 or 10 days. 6-week-old mice were treated with ATB-346 for 3, 7, or 10 days. At 14 weeks of age the mice were euthanized and polyp score was determined.

Intraperitoneal administration of ATB-346 and naproxen

Route of NSAID administration may alter the inhibitory effects of ATB-346. Therefore, we tested the effects of intraperitoneal administration of ATB-346 (14.5 mg/kg, i.p.) and naproxen (10 mg/kg, i.p.) on $Apc^{Min/+}$ mice. Six week old mice were treated daily for 14 days with naproxen, ATB-346 or Vehicle (95:5 CMC) intraperitoneally. At 14 weeks of age the mice were euthanized, and the small intestine and colon were removed and blindly scored for size and number of polyps/tumours.

The effect of ATB-346 and naproxen treatment on mice after the formation of polyps/tumours

To test the therapeutic potentials of ATB-346 we treated mice after the development of colonic and intestinal polyps/tumours.12 week old mice were treated orally with naproxen (10 mg/kg, p.o.), ATB-346 (14.5 mg/kg, p.o.), or vehicle (95:5, 1%CMC:DMSO) for 14 days. At 14 weeks of age the mice were euthanized and polyp score was determined.

The chemo-preventative effects of diallyl disulfide

We tested the chemo-preventative effects of a non-TBZ hydrogen sulfide donor, Diallyl Disulfide (DADS). 6-week-old $APC^{Min/+}$ mice were orally treated with DADS at 3, 10 or 30 $\mu mol/kg$ daily for 14 days. At 14 weeks of age the mice were euthanized and polyp score was determined.

Western blotting of ATB-346, naproxen, or vehicle-treated mice

Western blot analysis was used to determine expression of β-catenin, APC, p53, cmyc, NRF-2, Keap1, NQO1 and p53BP1. Samples were collected from mice treated with ATB-346, naproxen, vehicle and untreated C57/BL-6 mice. Mice tissue was processed and blots were prepared as described in section 2.5. Proteins were separated on 4–20% gradient polyacrylamide gels. Rabbit polyclonal anti-NRF2 (1:500), anti-Keap1 (1:500), anti-NQO1 (1:500) anti-p53BP1 (1:500), anti-β-catenin (1:500), anti-APC (1:500) anti-p53 (1:500), and anti-cmyc (1:500), were used (Cell Signaling Technology, Beverly, MA, USA). Enzyme expression was visualized using a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:1000) and an enhanced chemiluminescence detection kit on a Chemi-doc gel imaging system (Bio-Rad, Canada). The intensity of the bands was determined and analyzed using ImageLab 2.0 software (Bio-Rad, Canada). The expression of each enzyme was normalized to the expression of β-actin (Cell Signaling Technology, Beverly, MA, USA, 1:1000).

Photograph of small Intestine and colon of treated APC^(Min/+) mice

Mice colons and small intestines were photographed using a Nikon 3200 camera. Representative images of findings are presented.

4.3. Results

APC^(Min/+) Mice develop macroscopic polyps/tumours by 12 weeks of age

After euthanizing APC^(Min/+) mice at different ages we determined that these mice develop macroscopically visible polyps/tumours in the small intestine and the colon at 12

weeks of age. By 14 weeks of age there are many macroscopically visible polyps/tumours in the small intestines and the colon. These mice have enlarged spleens, and a deterioration of health. Some mice appeared to have a change in behavior and blood in stool. We used 14 weeks of age as an endpoint of the experiment.

ATB-346 significantly inhibited polyp/tumour formation

Wild type mice and mice treated with ATB-346 and naproxen had no changes in health by 14 weeks of age. However, several of the vehicle-treated APC$^{Min/+}$ mice appeared unhealthy, and many had changes in pigment on their tails and feet. One mouse was euthanized at 13 weeks of age due to the appearance of blood in stool, and unhealthy appearance. Once euthanized wild type mice had no visible polyps/tumours in the small intestine or colon and had no visible changes in internal anatomy. All of the naproxen and vehicle-treated mice had robust polyp/tumour formation throughout the small intestine. The polyps/tumours ranged in diameter from 1 mm to 4 mm and were found throughout the small intestines, but more predominantly in the terminal ileum (Figure 4.1). These mice also had visible enlargement of the spleen and one mouse from the vehicle-treated group had intussusceptions due to a polyp/tumour occluding the intestine. The naproxen and vehicle-treated mice also presented with several colonic polyps/tumours that were generally larger in size than the intestinal polyps/tumours ranging in diameter from 3 to 6 mm (Figure 4.1). These polyps/tumours were found throughout the colon including the cecum and did not predominate in any specific portion of the colon. In contrast to naproxen and vehicle-treated mice, the ATB-346-treated mice had no visible enlargement of the spleen or intussusceptions. 3 of the 4 mice treated with ATB-346 had no

polyps/tumours in the small intestine. The lone mouse that did have polyps/tumours had fewer and smaller polyps/tumours than the naproxen- and vehicle-treated mice (Figure 4.1). Furthermore, the ATB-346-treated mice did not develop any colonic polyps/tumours (Figure 4.1). Images taken of the small intestine and colon from the 4 groups illustrate the remarkable effect ATB-346 caused in the APC$^{Min/+}$ mice. When total polyp score was calculated, we found mice treated with ATB-346 had a significantly lower total polyp score compared to the vehicle-treated group (**p<0.001) (FIGURE 4.2).
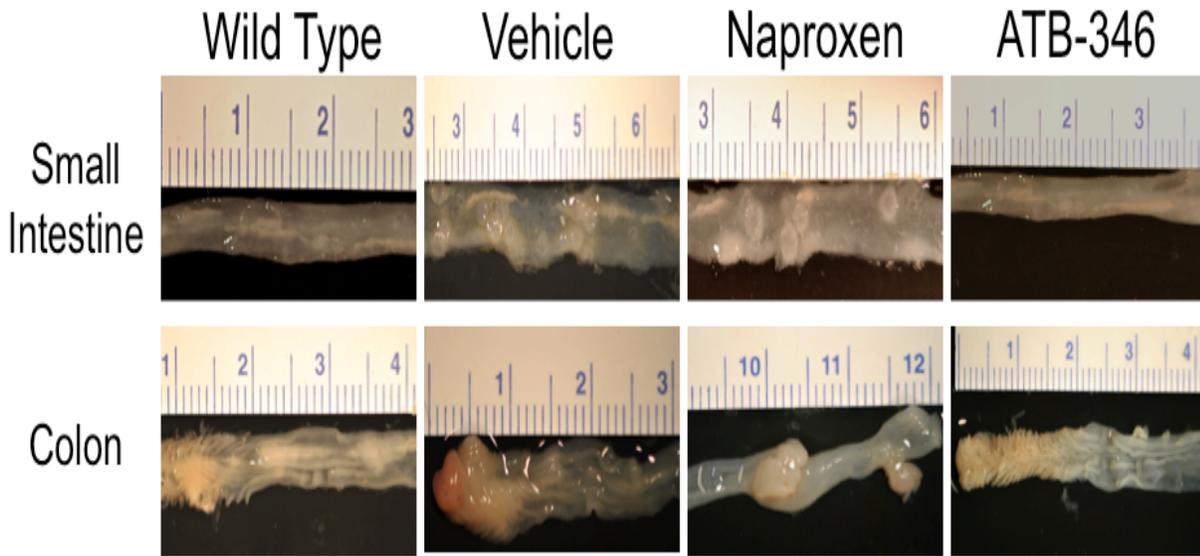
Figure 4.1: Photograph of small Intestine and colon of treated APC$^{Min/+}$ mice.

Polyps/tumours can be easily observed in mice treated with vehicle and Naproxen in the small intestine and colon. ATB-346-treated mice have no visible polyps/tumours in the small intestine or colon.
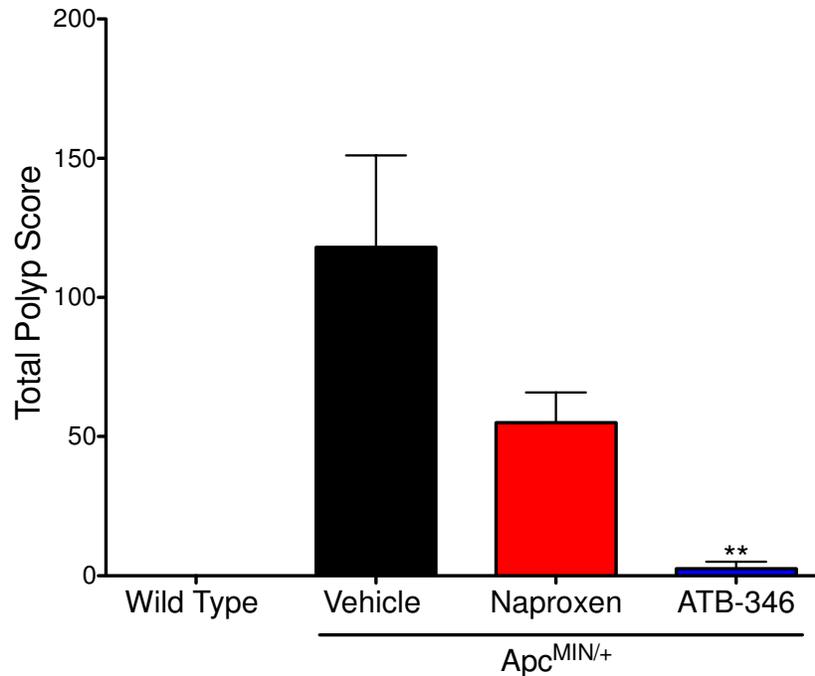
Figure 4.2: Reduction of total polyp score by ATB-346. APC[Min/+] mice treated with ATB-346 at 14.5 mg/kg (equimolar to 10 mg/kg of naproxen) for 14 days had a significantly lower total polyp score compared to vehicle-treated and naproxen-treated mice. Naproxen produced no statistically significant reduction in the total polyp score. ***p<0.0001 ATB-346-treated group vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 4 per group.

Ketoprofen and ATB-352 produced equivalent chemo-preventative effects

In this study we found that vehicle-treated and TBZ-treated mice developed abundant polyps/tumours in the small intestine and colon, similar to what was observed in the vehicle-treated mice in the previous study. TBZ-treated mice had equivalent total polyp score as vehicle-treated mice (FIGURE 4.3). Furthermore, mice treated with ATB-352 or ketoprofen also developed polyps/tumours in the small intestine and colon, however when total polyp score was calculated we found that both treatment groups had

significantly lower total polyp score compared to the vehicle-treated counterparts (**$p<0.001$) (Figure 4.3). Moreover, we did not find any significant difference between the mice treated with ATB-352 and those mice treated with ketoprofen.
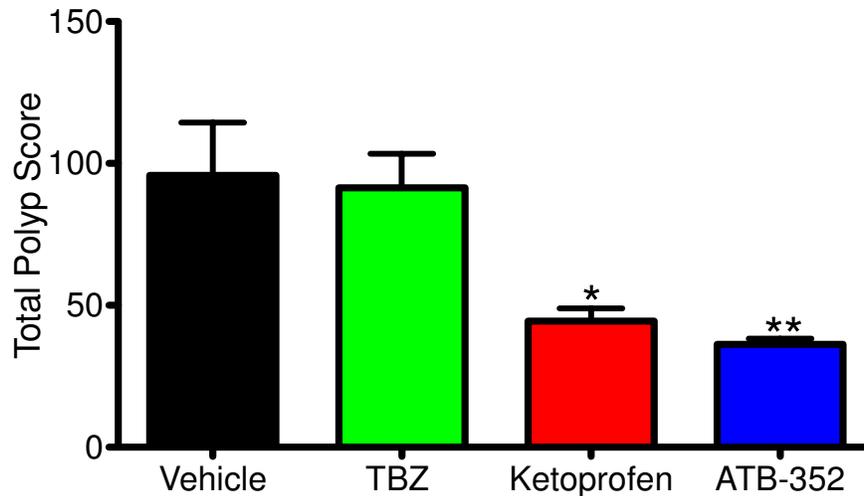
Figure 4.3: The effect of TBZ, ketoprofen or ATB-352 treatment in APC$^{Min/+}$ mice. Treatment of Apc$^{Min/+}$ mice with ketoprofen (10 mg/kg) or ATB-352 (15.2 mg/kg, equimolar to ketoprofen dose tested) caused a significant reduction in the total polyp score. However, treatment with TBZ caused no significant reduction in total polyp score. *$p<0.05$ ketoprofen-treated group vs. vehicle-treated group, **$p<0.01$ ATB-352-treated group vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 5 per group.

Treatment of ATB-346 for 3, 7, or 10 days caused a significant reduction in total polyp score

Treatment protocol often affects the therapeutic results of drugs. We tested ATB-346 at shorter periods of 3, 7, and 10 days. Mice treated with ATB-346 for 3 days developed polyps/tumours in the small intestine and colon, the multiplicity of

polyps/tumours was significantly less than that observed in the vehicle-treated mice. Although less, the localization and size of the polyps/tumours was not different than in the vehicle-treated mice (***p<0.05) (FIGURE 4.4). Mice treated for 7 or 10 days with ATB-346 also developed small intestinal polyps/tumours. However, there were significantly fewer polyps/tumours than what was observed in the vehicle-treated mice (***p<0.0001. Figure 4.4). Remarkably these mice developed no polyps/tumours in the colon similar to the observations with 14 days treatment of ATB-346 (*p<0.05. Figure 4.5). Total polyp score was significantly lower in mice treated with ATB-346 at all treatment lengths tested (***p<0.0001. Figure 4.4). Furthermore, we observed a significant reduction in total polyp score at 10 days of ATB-346 treatment compared to 7-day treatment (***p<0.0001. Figure 4.4).
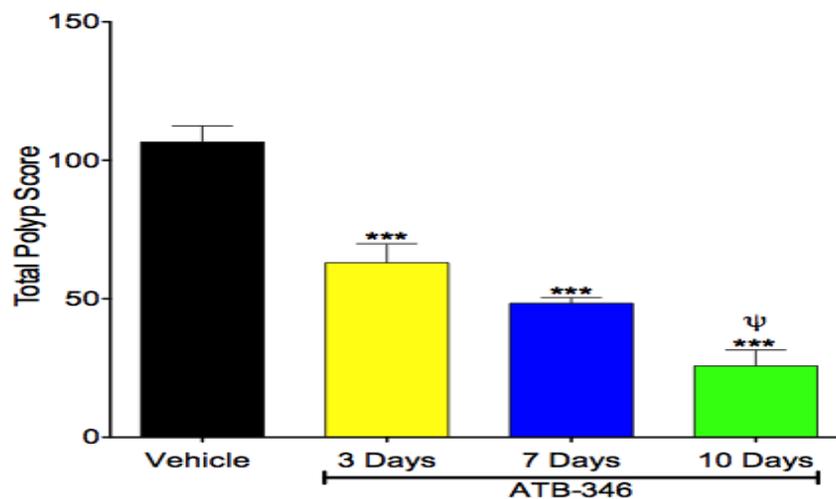
Figure 4.4: Reduction of total polyp score by 3, 7, and 10-day treatment of ATB-346. Apc$^{Min/+}$ mice treated with ATB-346 for 3, 7 and 10 days had significantly lower total polyp score compared to vehicle. Furthermore, 10-day treatment with ATB-346 caused a significant decrease in total polyp score compared to 3 days and 7 days of treatment. ***p<0.0001 3 day, 7 day and 10 day treated groups vs. vehicle-treated group. ψ p<0.01

10 day-treated group vs. 7 day-treated group. Data are expressed as mean ± S.E.M. n of 5 per group
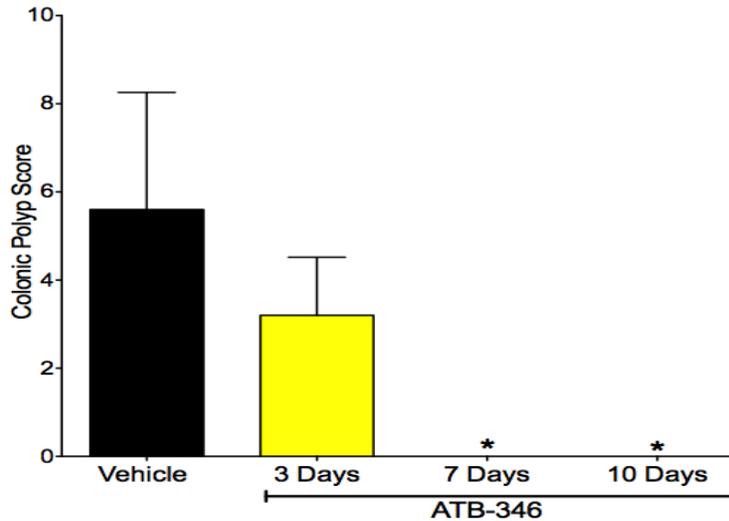
Figure 4.5: Reduction of colonic polyp score by 3, 7, 10 days of treatment with ATB-346. Apc$^{Min/+}$ mice treated with ATB-346 for 3, 7 and 10 days had significantly lower colonic polyp score compared to vehicle. *$p<0.05$ 7 and 10 day-treated group vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 5 per group.

Intraperitoneal treatment of naproxen and ATB-346 produced no changes in total polyp score of APC$^{Min/+}$ Mice.

Route of drug delivery may affect efficacy of chemo-prevention. We intraperitoneally administered ATB-346 and naproxen to determine if oral administration was necessary to produce chemoprevention. We found treatment with naproxen and ATB-346 (i.p.) caused no change in the number, size or distribution of polyps/tumours. These mice treated with ATB-346 and naproxen were unremarkable to vehicle-treated mice (Figure 4.6).
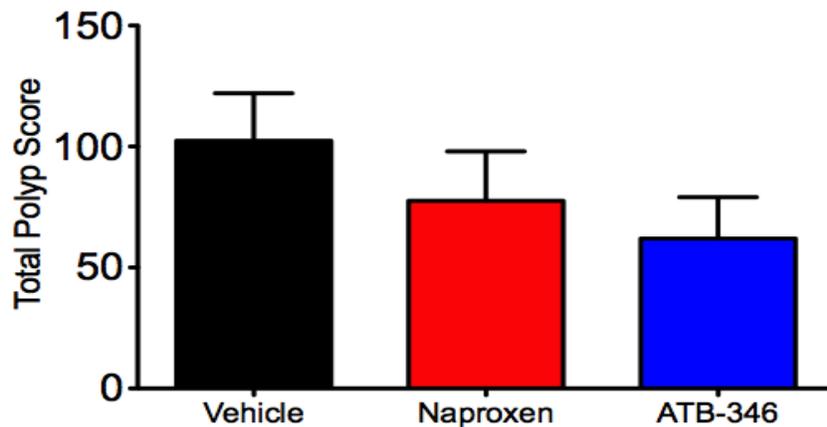
Figure 4.6: The effect of intraperitoneal treatment with ATB-346 on total polyp score. Intraperitoneal Treatment of Apc$^{Min/+}$ mice with naproxen (10 mg/kg) or naproxen (14.5 mg/kg, equimolar to naproxen dose) had no effect on the total polyp score. Data are expressed as mean ± S.E.M. n of 5 per group.

The effect of ATB-346 and naproxen on polyp/tumour formation of APC$^{Min/+}$ mice that began treatment at 12 weeks of age.

After determining that mice develop macroscopically visible polyps/tumours in the small intestine and colon by 12 weeks of age, we tested the therapeutic potentials of ATB-346 at the same dose and treatment protocol that we have found effective as a chemo-preventative. Mice treated with ATB-346 at 12 weeks of age had a plethora of polyps/tumours throughout the small intestine and colon in similar quantity to the vehicle-treated mice. The localization and size of polyps/tumours was unremarkable compared to the vehicle treated mice. We observed no significant difference in total polyp score between ATB-346-treated mice and vehicle-treated mice (FIGURE 4.7).
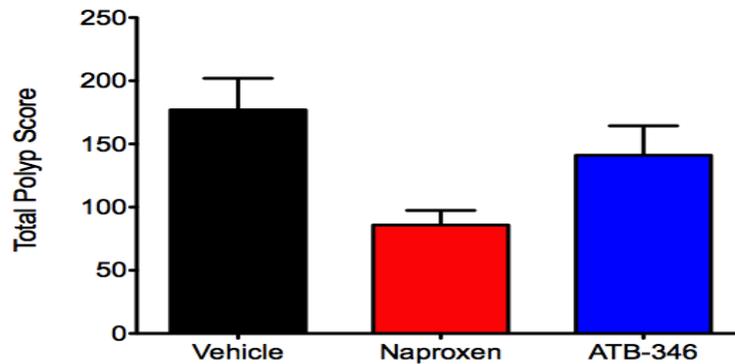
Figure 4.7: ATB-346 and naproxen produced no changes on total polyp score of APC$^{Min/+}$ that began treatment at 12 weeks of age. 12-week-old Apc$^{Min/+}$ mice treated with naproxen (10 mg/kg) or ATB-346 (14.5 mg/kg, equimolar to naproxen dose) for 2 weeks had no change in the total polyp score. Data are expressed as mean ± S.E.M. n of 5 per group.

Diallyl Disulfide reduced total polyp score in APCMin /+ mice

In this experiment we tested the ability of DADS, a donor of H_2S, to inhibit polyp/tumour formation in APC$^{Min/+}$ mice. Half of the mice treated with 30 μmol/kg of DADS died prior to reaching endpoint. The surviving mice had remarkable reduction in total polyp score. Unfortunately, due to the small sample size (n) it was not statistically significant. 10 μmol/kg of DADS led to a significant reduction in total polyp score (**$p<0.001$. Figure 4.8). However, we observed no reduction in total polyp score in mice treated with 3 μmol/kg of DADS (Figure 4.8).
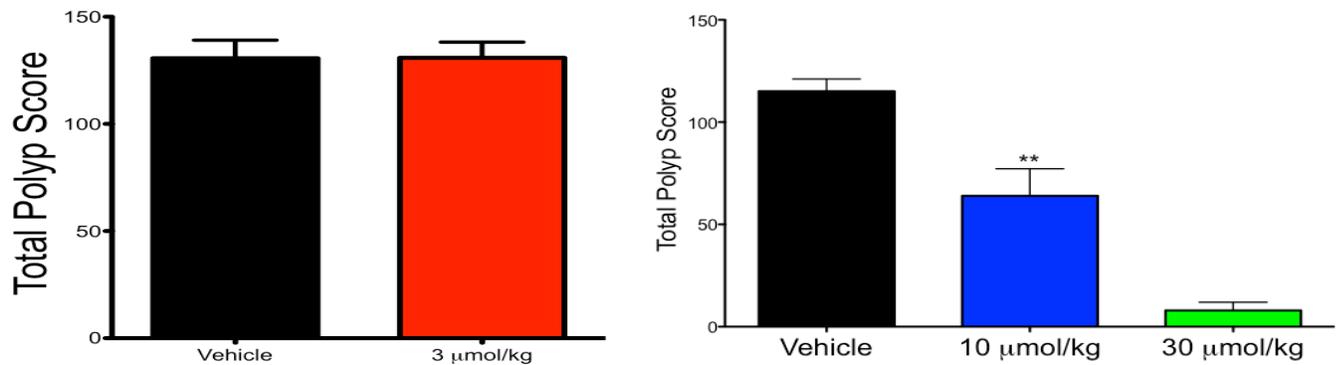
Figure 4.8: The effect of DADS on total polyp score. APC$^{Min/+}$ mice treated with 10 μmol/kg of DADS had a significant reduction in total polyp score compared to vehicle-treated mice. Treatment with DADS at 3 and 30 μmol/kg had no significant effect on total polyp score. **p<0.01 10 μmol/kg DADS vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 2 per group.

ATB-346-treated APC$^{Min/+}$ mice had higher expression of β-catenin and cmyc

To determine whether ATB-346 affects the Wnt pathway, western blotting was performed on APC, β-catenin, p53 and cmyc, which are all commonly mutated in human colorectal cancer. Western blots analysis indicates APC$^{Min/+}$ mice had higher levels of β-catenin and cmyc and lower levels of APC. Treatment with ATB-346 led to a significant decrease in cellular levels of β-catenin and cmyc (**p<0.001, *p<0.05, respectively. Figure 4.9A) and D). However, ATB-346 caused no significant changes in the levels of APC, or p53 (Figure 4.9B and C). Naproxen treatment led to no changes in any of the Wnt proteins tested.
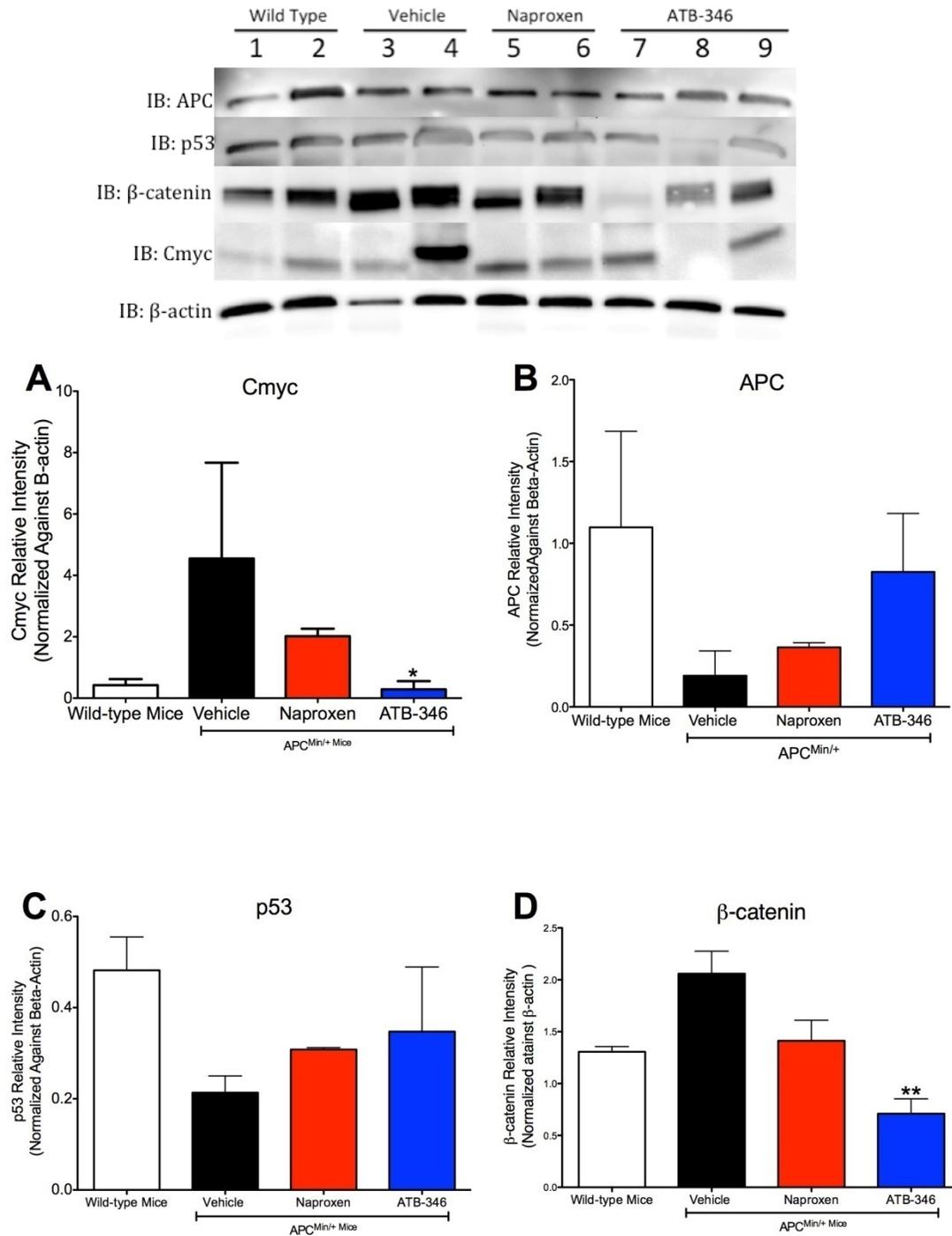
Figure 4.9: Reduction in β-Catenin, and cmyc levels in ATB-346-treated APC^Min/+ mice. Representative western blots showing protein levels of A) cmyc, B) APC, C) p53, D) β-catenin in intestinal tissue of mice treated with ATB-346, and vehicle. Treatment

with ATB-346 or naproxen caused a change in p53 or APC levels, But treatment with ATB-346 caused a significant reduction in cmyc and β-catenin expression. **p<0.01 ATB-346 vs. vehicle-treated group. *p<0.05 ATB-346 vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 4 per group.

ATB-346-treated APC$^{Min/+}$ mice had higher expression of Nrf2 and Keap1

A common mechanism associated with H_2S anti-cancer effects is it's antioxidant function. Using western blotting we tested the effect of naproxen and ATB-346 on several proteins associated with Nrf2 antioxidant pathway. APC$^{Min/+}$ mice had increased levels of Nrf2 and Keap1. ATB-346 treatment led to a reduction in Nrf2 and Keap1 levels (**p< 0.001, *p<0.05, respectively. Figure 4.10 and 4.11, respectively). However, ATB-346 caused had no significant effect on NQO1 or p53BP1 protein levels. Naproxen treatment led to no significant changes in Nrf2, Keap1, NQO1 or p53BP1 levels (4.12).
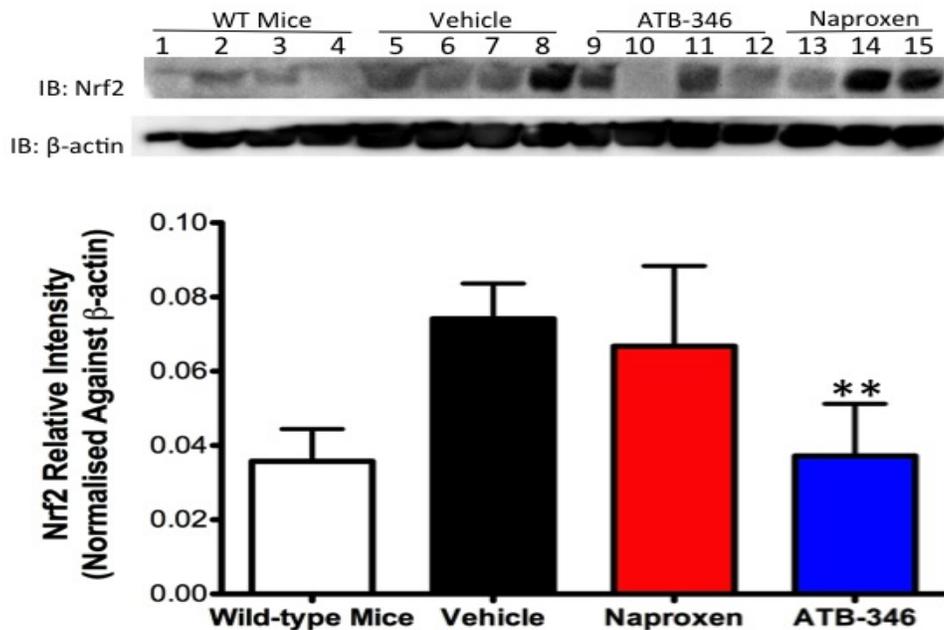
Figure 4.10: Reduction in intestinal Nrf2 levels in APC$^{Min/+}$ mice treated with naproxen or ATB-346. Representative western blot showing protein levels of Nrf2 in intestinal tissue are significantly lower in mice treated with ATB-346 compared to mice treated with vehicle. Naproxen treatment led to no significant changes in Nrf2 expression levels. **$p<0.01$ ATB-346 vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 4 per group.
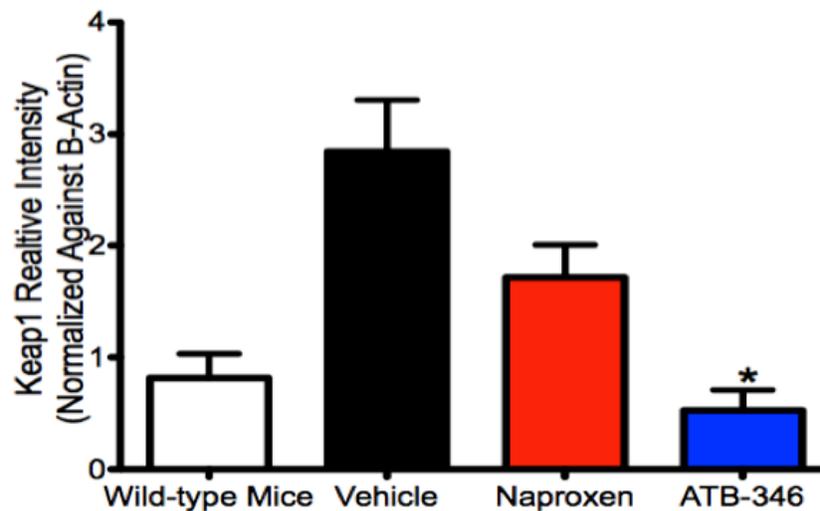
Figure 4.11: Reduction of intestinal Keap1 levels in ATB-346-treated APC$^{Min/+}$ Mice. Representative western blot showing protein levels of Keap1 in intestinal tissue are significantly lower in mice treated with ATB-346 compared to mice treated with vehicle. Naproxen treatment led to no significant changes in Keap1 expression levels. **$p<0.01$ ATB-346 vs. vehicle-treated group. Data are expressed as mean ± S.E.M. n of 4 per group.
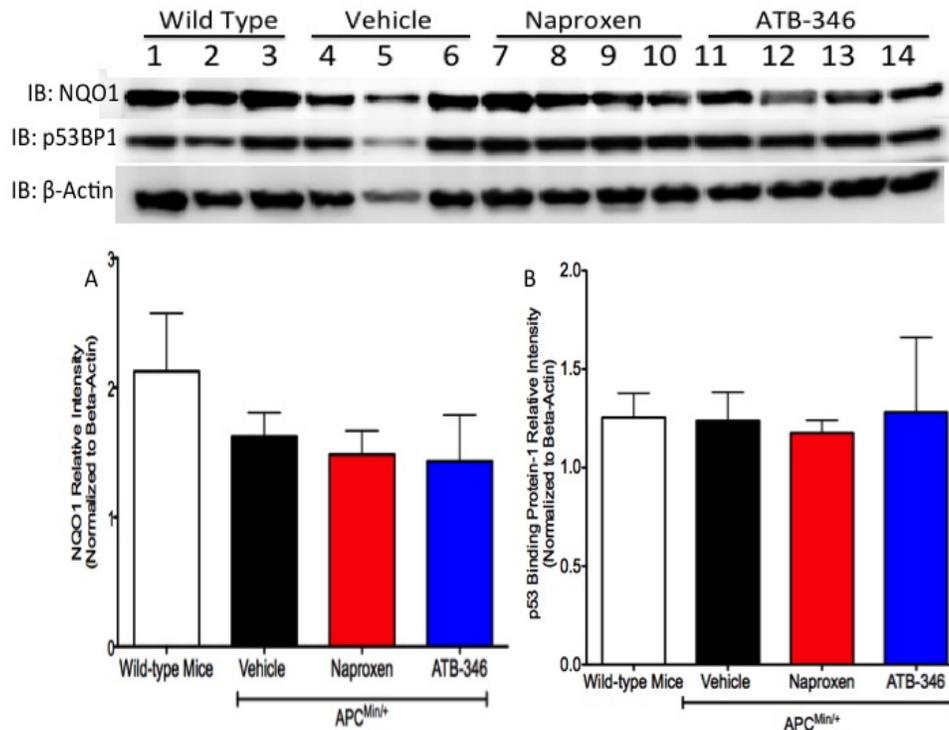
Figure 4.12: Effect of ATB-346 treatment on NQO1 and p53BP1 levels in APC$^{Min/+}$ mice. Representative western blots showing protein levels of NQO1 (A), and p53BP1 (B) in intestine. Data are expressed as mean ± S.E.M. n of 4 per group.

4.4. Conclusion

In this study we examined the chemo-preventative effects of ATB-346 (a H$_2$S-derivative of naproxen) and ATB-352 (a H$_2$S derivative of ketoprofen), and their parent compounds in an APC$^{Min/+}$ mouse model of colorectal cancer. We demonstrated the unique properties of ATB-346, by determining that TBZ does not cause any change in the number of polyps/tumours. Moreover, we observed equivalent chemo-preventative effects of ATB-352 and ketoprofen. In an attempt to understand possible mechanisms of the observed inhibitory effects of ATB-346 we analyzed the effects of ATB-346 on the Wnt pathway. We found that while ATB-346 does not affect APC levels within these mice it

significantly reduced the levels of β-catenin and cmyc, which are commonly up regulated in colorectal cancer. Ultimately, further research is required to further clarify the mechanism of ATB-346 action.

5.0 DISCUSSION

5.1. General Discussion

The present study provides evidence that, ATB-346 is a unique NSAID that provides a chemo-preventative effect unlike that of naproxen, ATB-352, ketoprofen or TBZ. ATB-346 significantly inhibited the formation of polyps/tumours in colorectal carcinoma. While the precise mechanism of ATB-346's inhibitory effects are still unclear several other reports have also presented similar findings in vivo and in vitro using H_2S donors and H_2S-NSAIDs. These studies have illustrated that H_2S-NSAIDs are potent inhibitors of cancer cell growth, proliferation, cell cycle progression and promoters of apoptosis of these cell lines (Chattopadhyay et al., 2012; Kodela et al., 2012). Furthermore, a recent study illustrated a Cox-independent chemo-preventative effect associated H_2S-NSAID inhibition of growth. They found that in cell lines that do express Cox-1 and Cox-2 H_2S-NSAIDs caused equivalent growth inhibition as in cell lines that do not express Cox (Farrugia & Balzan, 2013). A recent study further supports our results of H_2S-NSAIDs being a potent inhibitor of carcinogenesis in breast cancer (Frantzias et al., 2012). Chattopadhyay et al. have illustrated that H_2S releasing aspirin may suppress the growth of breast cancer cell lines by inducing cell cycle arrest, apoptosis and down-regulating NF-kB (2012).

The differential effect of NSAIDs on chemo-preventative efficacy is well noted (Sangha et al., 2005). It is likely that we did not observe superior reduction in the number of ACF by ATB-352 because of the different pharmokinetics. Differences in half-life, chemical structure, chemical activity and absorption greatly influence the chemo-preventative effects of individual NSAIDs. In addition to inhibiting cyclooxygenase

activity, ketoprofen also inhibits bradykinin and some lipoxygenases (KuKanich et al., 2012). Naproxen has been shown to undergo extensive enterohepatic recirculation resulting in prolonged half-life, an effect that is not observed with ketoprofen. These difference in pharmokinetics likely influence the difference in chemoprevention of the NSAIDs (Lobetti & Joubert, 2000).

Unlike our findings with TBZ, others have reported H_2S donors may induce anti-cancer activity in vitro. These findings are currently being disputed as reports have found that H_2S exerts both pro and anti-apoptotic activity in cell cultures (Cai et al., 2010; Lee et al., 2011a). The reason for this discrepancy may be associated with differences in H_2S donors, concentrations of H_2S, and experimental conditions. Na_2S and NaHS are widely used to study the biological effects of H_2S in many cells, tissues and animals. These sulfide salts generate abundant H_2S in a short period of time, which leads to a large concentration of H_2S within the cell lines (Cao et al., 2006). Studies using these salts often report a pro-proliferative function of hydrogen sulfide. Studies using GYY4137, which releases H_2S slowly over a period of hours to days, often report a pro-apoptotic and inhibition of tumour growth (Li et al., 2008). Neither GYY4137 nor sulfide salts have been reported to cause significant killing of non cancerous cells (Lee et al., 2011a).

In vivo studies of the therapeutic potentials of H_2S and H_2S-NSAIDs have focused on the use of tumour xenograft models (Chattopadhyay et al., 2012; Kashfi, 2009; Lee et al., 2011b; Piazza et al., 2009). In these models human tumor cells are transplanted subcutaneously into immunodeficient mice. The tumours are left to grow and the response to appropriate therapeutic can be studied in vivo. Recently, information regarding the key influence of the tumour microenvironment on tumour progression and growth has put this

model into question. The immune response to tumours is lost when using these models because immunodeficient mice do not possess a functioning immune system (Richmond & Su, 2013). Furthermore, this subcutaneous tumour model does not represent appropriate sites for human tumours and are not predictive to test response to cancer therapeutics (Richmond & Su, 2013). To determine clinical application of H_2S-NSAIDs one must use models that mimic the disease in humans and xenograft models to test drug responses do not correlate with clinical activity in human patients.

5.2. Future Directions

These findings illustrate the unique effects of ATB-346 in colorectal cancer. This novel NSAID may help in reducing the occurrence of colorectal cancer within the general population and in patients who are genetically susceptible. Although future research is required to elucidate the specific mechanism by which ATB-346 inhibited the formation of polyps/tumours these studies may help to ignite future research. With multiple cellular pathways affected by carcinogenesis there are many signaling cascades that ATB-346 may be involved in. We have only begun to uncover the potential anti-cancer activity of this novel drug.

Furthermore, future research may focus on other epithelial forms of cancer including breast, prostate and skin cancers. Since many of these cancer types share common signaling cascades and protein mutations it may be likely that ATB-346 induces similar effects.

Further understanding of the H_2S and its role in cancer will allow us to better understand the possible mechanism by which ATB-346 is exerting these chemo-

preventative effects. Future research may also investigate the association between

endogenous H_2S and colorectal cancer.

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APPENDIX

THIS THESIS IS A COMPILATION OF THE FOLLOWING MANUSCRIPTS:
ELSEIKH W, WALLCE JL. H_2S-Releasing Naproxen Derivative (ATB-346) Exhibits Superior Chemo-Preventative Effects in Murine Colorectal Cancer. *In Preparation,* 2013.