Activation of Guanylate Cyclase-C With Heat-Stable Enterotoxin Fails to Potentiate Celecoxib-Induced Reduction in Colorectal Cancer Cell Growth in Vitro

Activation of Guanylate Cyclase-C with Heat-Stable Enterotoxin Fails to Potentiate Celecoxib-Induced Reduction in Colorectal Cancer Cell Growth *in Vitro*

Samuel J. Sagartz

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Abstract

This study evaluated the use of heat-stable enterotoxin (ST), a guanylate cyclase receptor type C (GC-C) agonist, in combination with celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, for the prevention of colorectal cancer (CRC) growth *in vitro*. GC-C is a membrane-bound enzyme found in the lumen of the intestines and is responsible for the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Activation of GC-C elevates intracellular cGMP which controls fluid-ion homeostasis and enterocyte differentiation along the crypt-villus axis. GC-C function can regulate colonic cell proliferation and induce cell cycle arrest. COX-2 is a cytosolic enzyme that catalyzes the formation of prostaglandins from arachidonic acid and inhibition of this enzyme is known to inhibit proliferation of human cancer cells. Previous research demonstrated that inhibition of the primary enzyme responsible for degradation of cGMP potentiated the cytostatic effects of celecoxib. This thesis tested if GC-C activation can enhance the antiproliferative effects of celecoxib.

Cell proliferation was assessed using a thymidine incorporation protocol, in which cells are cultured with tritiated thymidine after treatment and the amount of measured incorporated radioactivity correlates with cell division. Celecoxib was found to inhibit proliferation of T84 cells, which are derived from a human colorectal adenocarcinoma. This was consistent with previous studies that demonstrated an anti-proliferative effect of celecoxib in other human cancer cell lines. However, the addition of ST to celecoxib was

unable to potentiate the anti-proliferative effects of celecoxib. ST also had no effect on proliferation when used as a monotherapy.

Changes in COX-2 activity, as well as the presence of COX-2 enzyme, were measured to assess whether the effect of celecoxib in T84 cells was dependent on its inhibitory activity on COX-2. No changes in cyclooxygenase activity were observed and COX-2 was not detected in T84 cells. In addition, celecoxib and the combination of celecoxib and ST were unable to produce a detectable amount of Caspase-3, a marker of cell death, suggesting that these drugs do not have a cytotoxic effect in T84 cells.

This research showed that ST does not have an effect on proliferation of T84 cells, which conflicts with previous research conducted by Pitari et al. (2001). It is possible that an effect of ST on adenocarcinomas in the gastrointestinal tract may vary based on the location of the targeted cells. Therefore, while ST did not reduce proliferation on cells derived from the colon, it may have an effect on proliferation of epithelial cells that are located in the small intestine upstream from the colon. Celecoxib was confirmed to have an anti-proliferative effect, and this was shown to be independent of the drugs enzymatic target, implying that there are additional mechanisms through which celecoxib exerts its activity. Additional research is needed to clarify these mechanisms.

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Chapter I

Introduction

Colorectal Cancer

Colorectal cancer (CRC) is the third most common form of cancer worldwide, after lung and breast cancer (Singh, Montalban, & Mahmud, 2014). Accounting for approximately 600,000 deaths worldwide in 2008, CRC is also the fourth most common cause of cancer deaths, after lung, stomach, and liver cancer, and is the second most common cause of cancer death in developed countries. While CRC incidence and mortality rates have been declining in most developed countries over the past two decades (Yau et al. 2008), rates have actually been climbing in developing countries. With growing elderly populations across the world, even developed countries are likely to see an increase in the total number of patients diagnosed with CRC.

Nearly all colorectal cancers are adenocarcinomas (Bardhan & Liu, 2013). Most colorectal cancers begin with the development of polyps on the epithelial lining of the large bowel. Polyps may initially be benign growths, commonly referred to as hyperplastic polyps. Pre-malignant polyps, or adenomas, are the next step in progression to CRC. CRC typically develops slowly, over the course of many years. During this time, polyps or adenomas can progress to a malignant state, at which point they are termed adenocarcinomas.

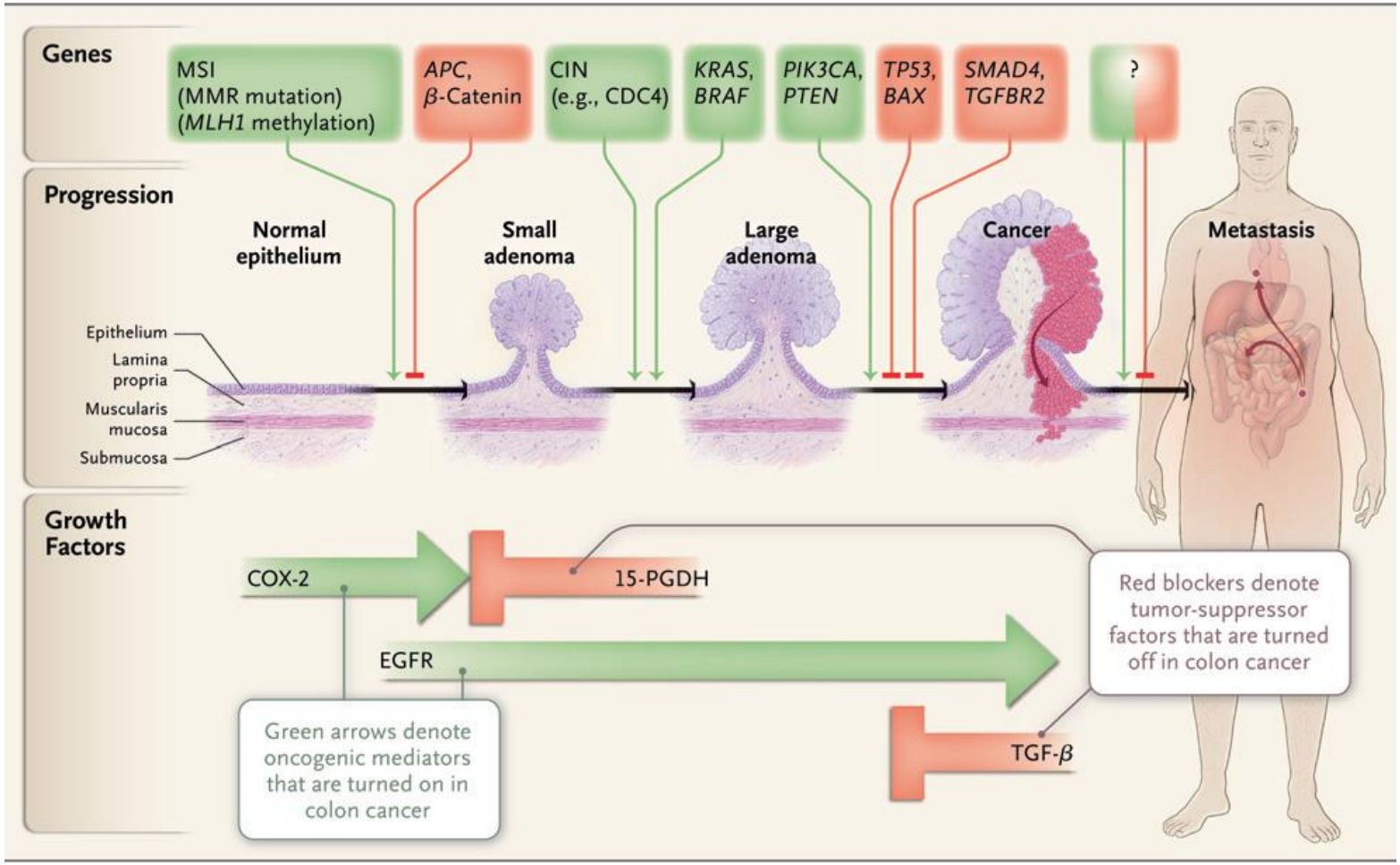
While about 20% of CRC patients have a family history of CRC, the majority of cases are linked to environmental factors, particularly diet and lifestyle. Researchers have

recently identified additional CRC risk factors including the composition of the intestinal microbiome and the presence of intestinal inflammation (Zhu, Gao, Wu, & Qin, 2013). Aberrant cellular activities at a variety of levels can eventually lead to progression of luminal intestinal tissue from normal glandular epithelium to adenocarcinomas (Markowitz & Bertagnolli, 2009). At the chromosomal level, CRC is associated with many rare inactivating mutations of genes that normally maintain chromosomal stability. In some patients, genes required for DNA repair (mismatch-repair genes) are inactivated. This inactivation can be inherited, or may result from epigenetic silencing as a consequence changes in DNA methylation. Epigenetic silencing can similarly result in a lack of chromosome-stabilizing genes.

The importance of epigenetic factors in CRC development has become increasingly understood over the last several decades (Bardhan & Liu, 2013). While the term epigenetics can be used to describe several non-coded mechanisms for altering DNA expression, changes in chromatin conformation appear to be particularly relevant in cancers in general and CRC in particular. By altering the extent to which DNA is accessible to transcription factors, epigenetic variations can lead to differences in activation or repression of transcription. In the CRC setting, it appears that variations in DNA methylation and modifications to histones proximal to gene promoters are the predominant epigenetic factors contributing to CRC development.

Chromosomal instability, DNA-repair defects, or altered DNA methylation can dramatically accelerate the accumulation of DNA mutations that can eventually lead to the development of CRC. Mutations in the Wnt pathway are particularly common in CRC tissue (Bardhan & Liu, 2013). Specifically, mutations in the *adenomatous polyposis coli*

gene (*APC*), which are observed in 85% of CRC (Markowitz & Bertagnolli, 2010), lead to activation of the Wnt pathway and polyp formation. Subsequent mutations to the proto-oncogenes *KRAS* or *BRAF* (via MAPK activation) or to T*P53*, β-*Catenin*, *SMAD4*, TGFβ receptor II (*TGFBR2*), or several other genes appear to facilitate progression from polyp to cancer by inducing proliferation and suppressing apoptosis. While these appear to be the most common etiologies in CRC pathogenesis, the molecular basis of the disease is not entirely understood, and numerous less prevalent mutations are also implicated in the pathogenic process (Bardhan & Liu, 2013).



Adapted from (Markowitz & Bertagnolli, 2009).

Figure 1. Factors Contributing to Progression of CRC.

Once a polyp develops into an advanced adenoma or early carcinoma, various growth and stem-cell factors can contribute to growth and metastasis of the cancer (Markowitz & Bertagnolli, 2010). An early step in adenoma growth is activation of prostaglandin signaling and growth factor signaling, which can occur in response to inflammation or increased expression of cyclooxygenase 2 (COX-2). Epidermal growth factor (EGF), which acts through the EFG receptor (EGFR) and the MAPK pathway, causes trophic effects that can promote metastasis. The angiogenesis-inducing vascular endothelial growth factor (VEGF) also promotes growth and spreading of tumors that are early in their development.

Certain individuals are heavily predisposed to polyp development and subsequent adenocarcinoma due to an inherited disease caused by specific genetic factors. One such disease is familial adenomatous polyposis (FAP). FAP is caused by mutations in the *APC* tumor suppressor gene which lead to constitutive β-catenin activity and subsequent hyper-proliferation (Plawski et al., 2013). As a result of this proliferation, individuals with FAP can develop hundreds of polyps, which typically begin to appear around age 15. With such an overabundance of polyps, these patients are at considerably higher risk of developing CRC. Individuals with mutations in the *MUTYH* gene may present with a similar phenotype; this autosomal recessive disease is referred to as MUTYH adenomatous polyposis (MAP).

Lynch Syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC), is the most common form of inherited susceptibility to CRC (Sehgal, Sheahan, O'Connell, Hanly, Martin, & Winter, 2014). Unlike FAP or MAP, however, HNPCC describes a collection of mutations to DNA mismatch repair syndromes. Also unlike the polyposis

described above, HNPCC does not necessarily lead to a massive number of polyps. The risk of CRC in individuals with HNPCC results mainly from the enhanced risk of progression from an adenoma to a carcinoma in patients with the disease. In fact, virtually every adenoma in HNPCC patients will progress to a carcinoma. In individuals without HNPCC, only approximately 1 in 30 adenomas will become a carcinoma.

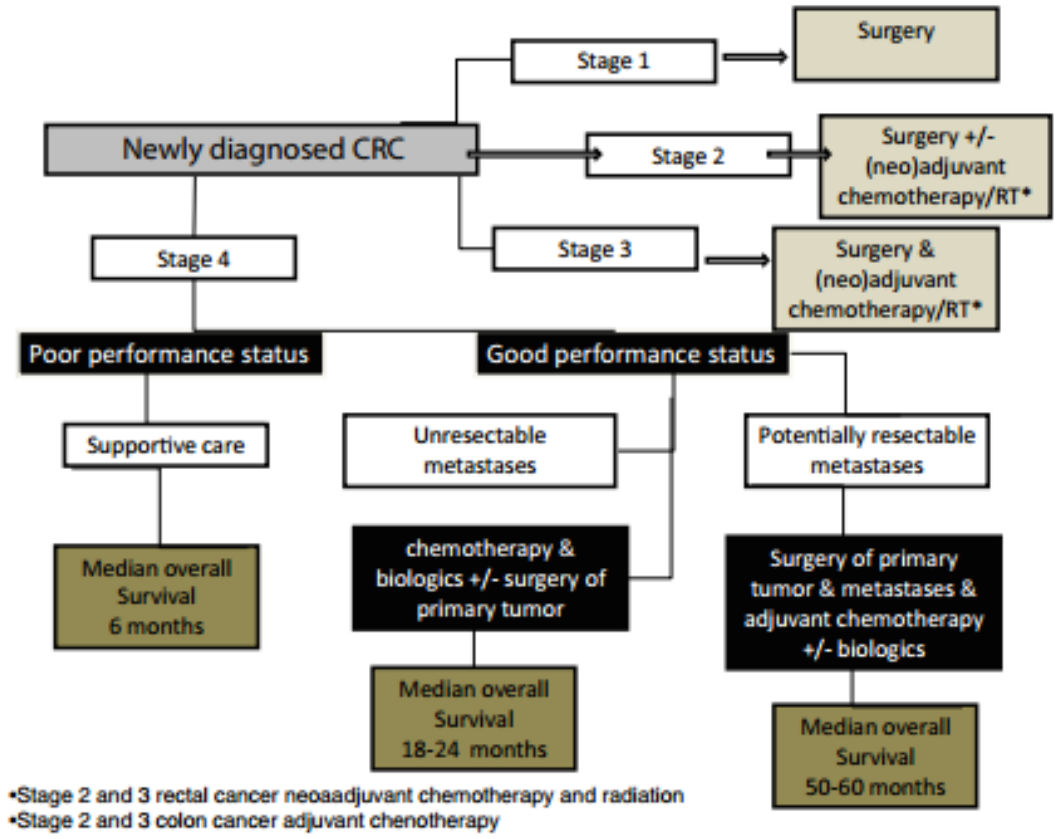
Treatment and Prevention of CRC

Localized or advanced colorectal carcinomas require surgical removal of the tumor (Courtney, McDermott, Heeney, & Winter, 2014). Surgical or endoscopic interventions substantially reduce recurrence and increase 5-year survival rates and are the mainstay of treatment in all CRC patients, with the exception of those with metastatic disease (Ahmed, Johnson, Ahmed, & Iqbal, 2014).

Postoperative chemotherapy is generally indicated in patients with advanced disease in which the cancer has been detected in neighboring lymph nodes (Tol & Punt, 2010). In these patients, chemotherapy reduces the likelihood of CRC recurrence and increases cure rates. However, adjuvant chemotherapy in patients whose cancer has not yet spread to lymph nodes is controversial. Adjuvant chemotherapy regimens have historically used the thymidylate synthase (TS) inhibitor 5-fluorouracil (5-FU) as the base of treatment. Levamisole, leucovorin, capecitabine, oxaliplatin, and numerous other compounds have since been added to the CRC treatment algorithm. Recent approvals of biological therapies including the anti-VEGF antibody, bevacizumab, and the anti-EGFR antibodies, cetuximab and panitumumab, have given physicians additional options for

treating advanced and metastatic CRC. Recent evidence also suggests that changes in lifestyle factors, such as diet and exercise, can improve the prognosis in CRC patients after resection of their tumor(s) (Meyerhardt et al., 2013).

In spite of the recently expanded arsenal of CRC treatments, 5-year survival in patients with distant metastases is less than 10% (Ahmed, Johnson, Ahmed, & Iqbal, 2014). As is the case when managing many other cancers, early detection and prevention of malignant growth and subsequent metastases are the primary goals of CRC treatment. The development and approval of new chemotherapeutic agents that prevent tumor growth and metastasis could substantially improve outcomes for patients with CRC. In certain patient populations, such as those with early polyps, significant genetic predisposition towards CRC, or those with FAP, early chemoprevention of further polyp growth with drugs such as aspirin may delay the development of a carcinoma.



Adapted from (Ahmed, Johnson, Ahmed, & Iqbal, 2014).

Figure 2. Framework for Managing Colorectal Cancer.

In patients with FAP or HNPCC, aspirin has not been found to produce a statistically significant reduction in polyp number, although it may reduce the size of polyps. On the other hand, a meta-analysis of four studies in individuals with previous history of adenomas or CRC found that aspirin did, in fact, provide a statistically significant 21% reduction in the relative risk (RR) of developing a recurrent adenoma(s). Furthermore, high dose aspirin treatment was found to reduce CRC incidence by a statistically significant 26% over a 23-year follow-up period. Interestingly, this effect was not detected over the first 10 years (Cooper et al., 2010).

Certain nonsteroidal anti-inflammatory drugs (NSAIDs) other than aspirin, such as sulindac, celecoxib, and tiracoxib, have also been evaluated in their ability to prevent adenoma or CRC incidence (Cooper et al., 2010). The studies evaluating these drugs in FAP patients have been very small. Nevertheless, five studies found that they were able to reduce polyp number and size. Celecoxib has also been evaluated in patients with history of adenomas over a 3-year follow-up period. Prevention with celecoxib was associated with a statistically significant 34% reduction in the RR of adenoma recurrence and a 55% reduction in the RR of incidence of advanced adenomas.

There appears to be a beneficial effect of chronic use of aspirin and other NSAIDs in reducing adenoma and CRC incidence (Cooper et al., 2010). Such use may be limited, however, due to concerns about the safety profiles of these types of drugs. For instance, aspirin is associated with gastrointestinal bleeding and other GI toxicities and some inhibitors of cyclooxygenase-2 have been associated with increased risk of serious cardiovascular events. This topic is discussed in more depth below. Because of these less-than-ideal safety profiles, it is worthwhile to investigate the use of other drugs that may be used in combination with NSAIDs to prevent CRC. The desired preventative effect would ideally be achieved using sub-toxic doses of both drugs. One potential approach for slowing CRC progression that has been described by certain academic groups is activation of the enzyme Guanylate Cyclase C, which has been shown to suppress intestinal inflammation and colon cell proliferation.

Guanylate Cyclase-C (GC-C)

Guanylate, or guanylyl, cyclases are a highly conserved family of enzymes that catalyze the conversion of cytosolic guanosine triphosphate (GTP) to the second messenger cyclic guanosine-3',5'- monophosphate (cGMP) in response to a variety of signals, depending on the specific guanylate cyclase (Lucas et al., 2000). cGMP was first identified in 1963 and the enzymes that catalyze its formation from GTP (i.e. the guanylate cyclases) were discovered 6 years later (Potter, 2011). cGMP modulates a variety of many physiological activities including neurotransmission, blood pressure, platelet aggregation, and fluid secretion. In the decades since cGMP and the first guanylate cyclase were discovered, eleven total guanylate cyclases, including four soluble proteins and seven membrane-spanning forms, have been found to be expressed in mammals.

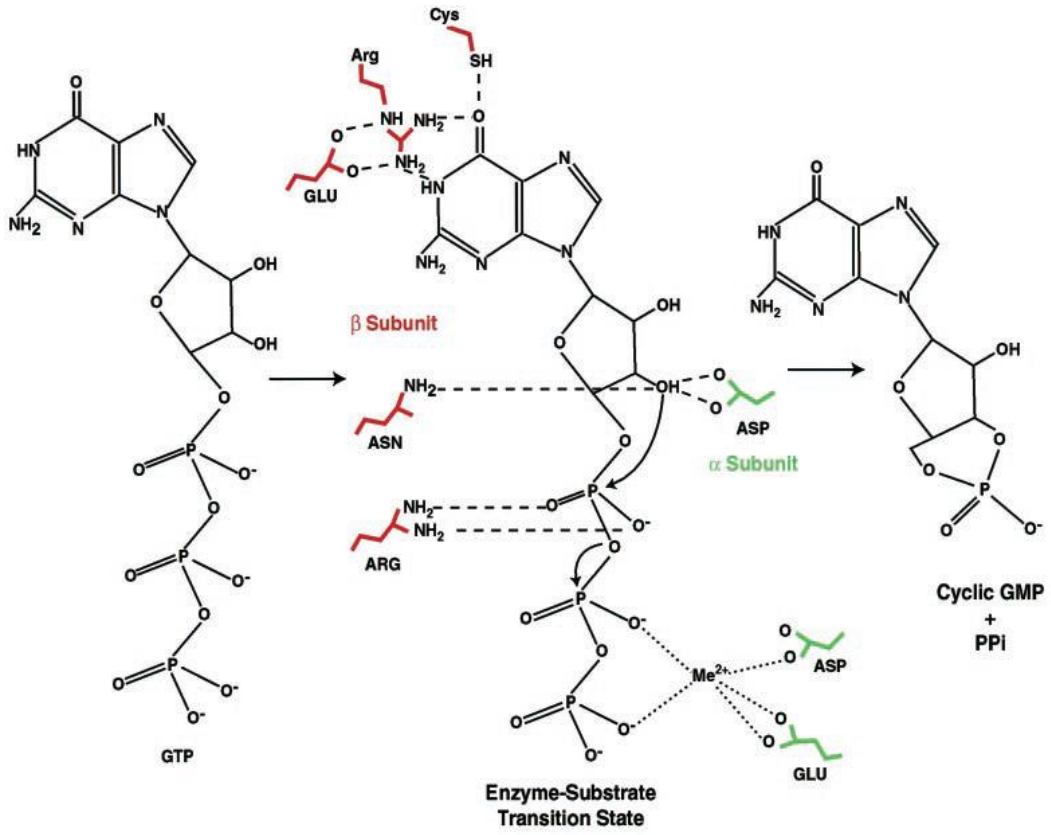


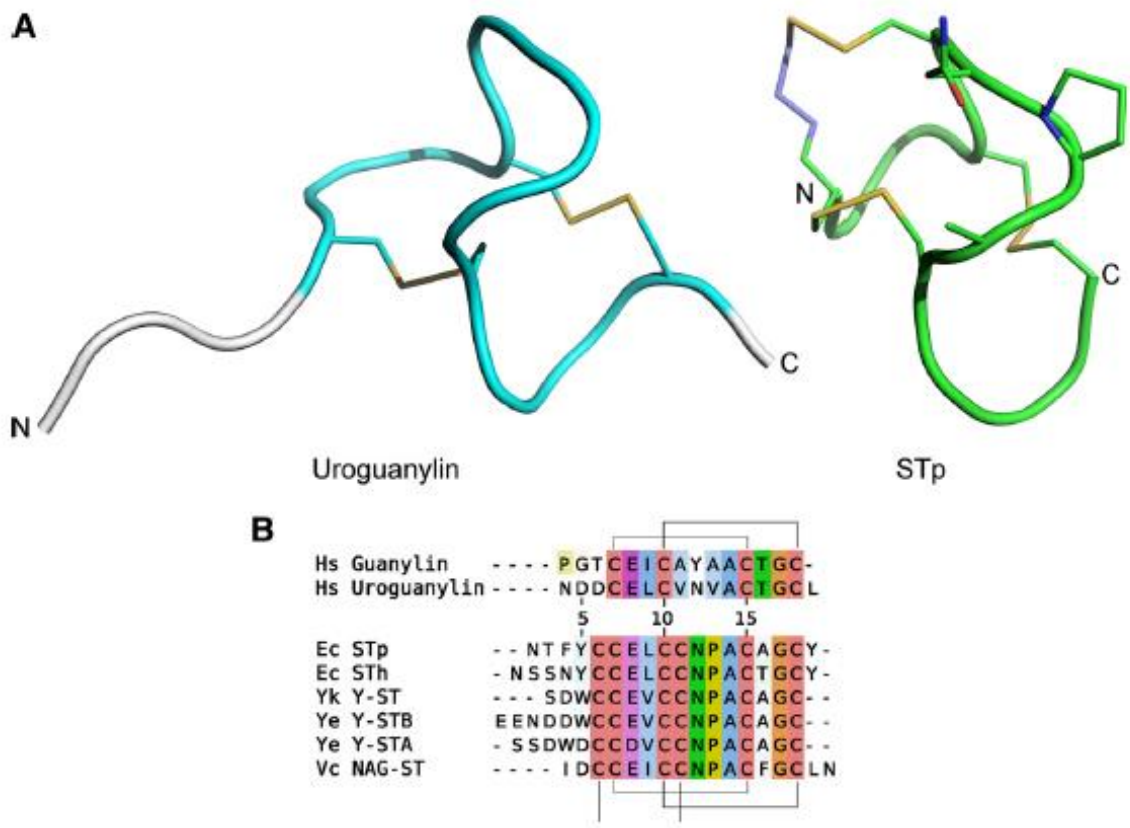
Image adapted from (Lucas et al., 2000)

Figure 3. Catalytic Mechanism of Guanylate Cyclase Activity.

GC-C converts linear GTP to cyclic GMP.

Guanylate cyclase C (GC-C) is expressed primarily in the intestinal epithelium, and is found in the apical membrane of gut epithelial cells (Potter, 2011). The extracellular ligand binding domain is coupled to the intracellular carboxyl terminal guanylate cyclase domain by a transmembrane region and an intracellular kinase homology domain. The catalytic domain is homologous to that of adenylyl cyclase, and is highly conserved across the guanylate cyclase family (Lucas et al., 2000).

The endogenous paracrine peptide hormones, guanylin and uroguanylin, as well as the heat-stable enterotoxin (ST), which is produced by various intestine-colonizing bacteria, bind to and activate GC-C, causing an increase in intracellular cGMP, which plays several roles in regulating cellular physiology (Arshad & Visweswariah, 2012). While guanylin and uroguanylin both bind GC-C in the lumen of the gastrointestinal tract, the two peptides differ in terms of their structure, stability and therefore, their cGMP-stimulating potency in different parts of the gut. Whereas uroguanylin is approximately 100-fold more potent than guanylin at pH 5.0, the relative potencies are reversed at an alkaline pH of 8.0 (Nakazato, 2001).



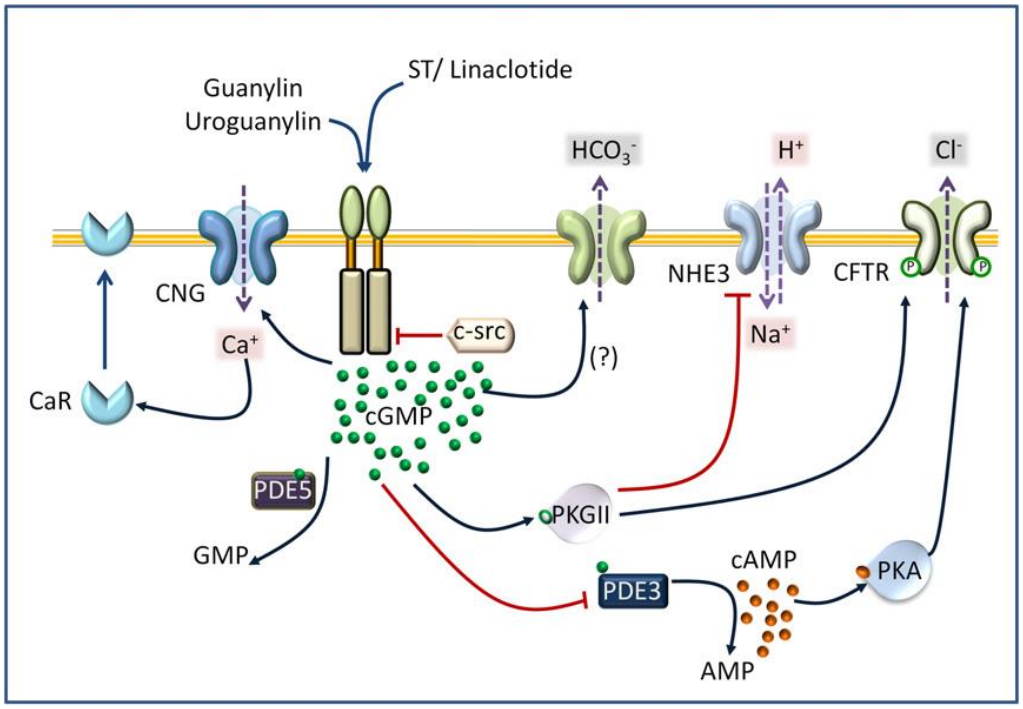
Adapted from (Taxt, Aasland, Sommerfelt, Nataro, & Puntervoll, 2010).

Figure 4. Structure and Primary Sequences of GC-C Agonists.

(A). Structures of human uroguanylin and the active domain of ST. (B). Amino acid sequences of various GC-C agonists.

In response to GC-C activation by guanylin, uroguanylin, ST, or other GC-C agonists, cGMP accumulates and activates cGMP-dependent protein kinase II (PKG II), which phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Arshad & Visweswariah, 2012). cGMP also inhibits the activity of PDE3, a cAMP-specific phosphodiesterase, resulting in activation of PKA. PKA phosphorylates the CFTR channel as well. CFTR phosphorylation results in a

continuously open structure, which allows chloride ions to flow out of the cell into the intestinal lumen. PKGII also leads to the efflux of bicarbonate ions and causes the inhibition of the sodium-hydrogen exchanger, NHE3 (Arshad & Visweswariah, 2012). These activities, coupled with CFTR phosphorylation, lead to a net reduction in sodium ion absorption and net fluid secretion into the intestinal lumen.



Adapted from (Arshad & Visweswariah, 2012).

Figure 5. Summary of GC-C Signaling.

Activation of GC-C leads to an increase in intracellular cGMP, which ultimately leads to chloride and bicarbonate secretion in the intestinal lumen, which causes net fluid secretion into the gut.

Linaclotide, a peptide with 14 amino acids contains 3 disulfide bonds, is a potent GC-C agonist approved in the United States and several other countries for constipation-predominant irritable bowel syndrome (IBS-C) and chronic idiopathic constipation (CIC) (Busby et al., 2012). Linaclotide has been shown to improve both motility and pain symptoms in patients (Quigley et al., 2013). Multiple other GC-C peptide agonists, including IW-9179, plecanatide, and dolcanatide (the latter two are uroguanylin analogs), are currently being developed for several indications including opioid-induced constipation, gastroparesis, and ulcerative colitis.

Whereas ST-producing bacteria utilize GC-C biology to manipulate host physiology and enhance transmission, mammals rely on guanylin- and uroguanylin-induced GC-C activation to maintain intestinal homeostasis (Arshad & Visweswariah, 2012). In addition to regulating fluid and ion secretion, GC-C-mediated cGMP increases have been shown to play a role in suppressing intestinal inflammation and colon cell proliferation.

GC-C and CRC

It has been suggested that GC-C acts as a cell cycle regulator and that activation of the enzyme suppresses tumor formation in the intestines. The colonic epithelia of GC-C-null mice has been found to be more susceptible to develop tumors in response to carcinogens or inherited germ line mutations compared to that of wildtype mice (Basu et al., 2014). Li et al. (2007) examined tumorigenesis in mice expressing GC-C and in GC-C-knockout mice. Some mice in each group had mutations in the *Apc* ($Apc^{Min/+}$) gene,

leading to rapid formation of polyps. In the $Apc^{Min/+}$ mice that were also deficient in GC-C, the researchers found increased tumor incidence in the colon compared to those expressing GC-C. Increased tumorigenesis was also observed in the small intestine of the GC-C knockout mice. These results were duplicated in non-$Apc^{Min/+}$) mice who were instead exposed to azoxymethane, a potent carcinogen. The authors also noted that there was an increase in number, but not size, of tumors in the colons of GC-C knockout mice compared to those expressing GC-C, suggesting that GC-C may not affect proliferation in the mouse colon, but instead may impact tumor differentiation via DNA damage. Increased DNA damage, measured using γ-H2AX, was observed as well, supporting this hypothesis. The authors concluded that the tumorigenesis observed in the colons of Apc^{Min+} mice lacking GC-C was the result of corrupted genomic integrity, not hyperproliferation. Chromosomal instability or impaired DNA repair, for example, may be the primary drivers of carcinogenesis in the large intestine.

While Li et al. (2007) suggest that genomic changes, instead of disruption of cell cycle checkpoints, are responsible for tumorigenesis in the colon, the authors propose that the story is reversed in the small intestine. In the same GC-C deficient $Apc^{Min/+}$ mice, tumor size, rather than number, compared to mice expressing GC-C appeared to be the driver of the increased tumor burden in this part of the gastrointestinal tract. Increased proliferation of enterocytes was also observed and was found to be associated with increased expression of β-catenin. Genomic abnormalities were not detected in the small intestine. Therefore, it appears that GC-C may suppress tumor development and growth via two distinct mechanisms that act in different parts of the gastrointestinal tract.

Additional evidence of a potential role of GC-C in colorectal cancer came from studies showing ectopic GC-C expression in metastatic colorectal tumors (Carrithers et al., 1996; later supported by Birbe et al., 2005; Schulz et al. 2006). Soon after, the guanylate cyclase C agonists ST and uroguanylin were found to have strong, dose-dependent cytostatic effects in two human colon carcinoma cell lines, T84 and Caco2, which express GC-C (Pitari, Di Guglielmo, Park, Schulz, & Waldman, 2001). Importantly, this inhibition of proliferation was not observed in SW480 cells, which do not express GC-C. The cytostatic effects were mimicked by a cell-permeant cGMP analog, and were potentiated by inhibiting the cGMP-specific phosphodiesterase inhibitor (PDE5). Furthermore, an inactive analog of ST failed to produce similar effects. These data strongly suggest that the observed cytostasis in T84 and Caco2 cells was mediated by GC-C catalyzed cGMP accumulation (Pitari, Di Guglielmo, Park, Schulz, & Waldman, 2001).

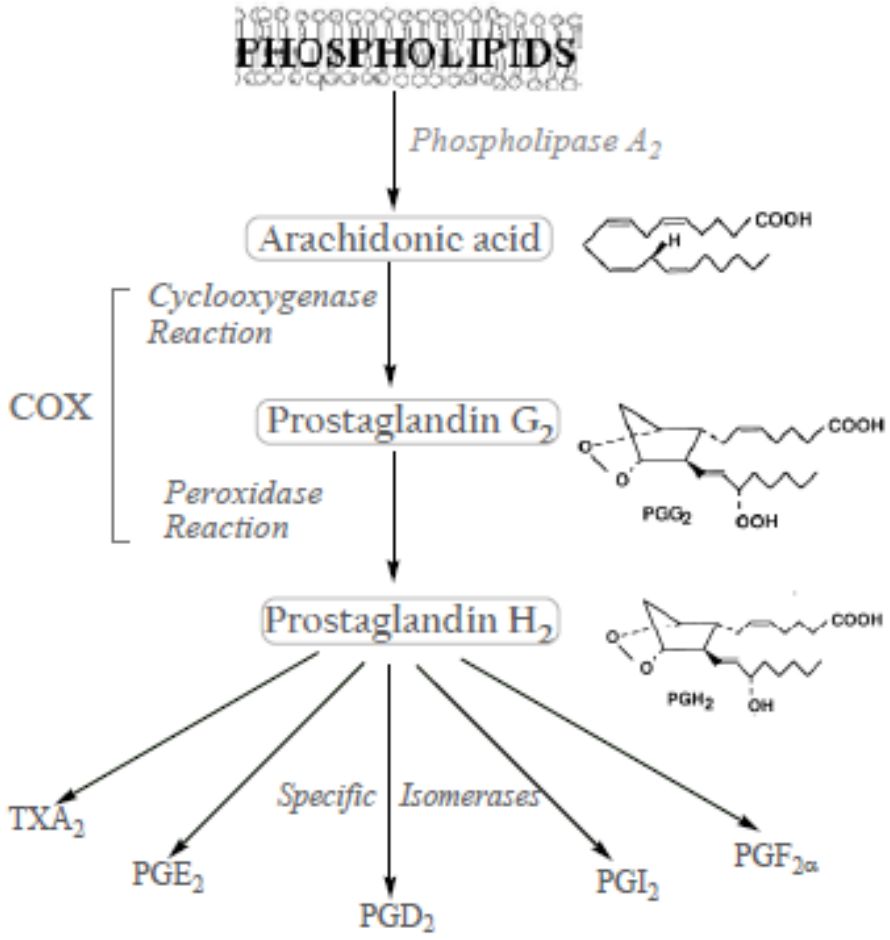
Wilson et al. (2014) recently assessed the expression of guanylin in CRC cells to see if a change in the expression of this GC-C agonist is associated with CRC. Guanylin mRNA and guanylin protein were found to be significantly reduced in CRC tumors when compared with normal epithelial specimens. Guanylin mRNA was reduced 100- to 1,000-fold in nearly all of the tumors compared with adjacent epithelia. Guanylin, which was detected in all 30 normal specimens, was found in none of the 54 tumors examined. These data suggest that the loss of guanylin and the presumed decrease in GC-C expression and activity may disrupt intestinal homeostasis, potentially leading to tumorigenesis.

If diminished GC-C activity is at least partially responsible for tumorigenesis or proliferation, it follows that GC-C activation may suppress malignant activities, as was initially shown by Pitari et al. (2001). More recent studies have suggested potential mechanisms for such neoplastic inhibition. Lubbe et al. (2009) showed that GC-C-mediated cGMP signaling leads to a redistribution of MMP-9, resulting in diminished capacity for the cytoskeleton reorganization that contributes to the metastasis of CRC and other cancer types (Powell, Bennett, Orange, Horgan, & Edwards, 2012; Chou et al., 2012). These mechanistic findings were supported by reduced establishment of CRC metastases in mouse peritoneum *in vivo*. Lin et al. (2010) suggested that inhibition of Protein Kinase B, otherwise known as AKT, might be another potential mechanism for GC-C-induced tumor suppression. AKT has been suggested as an inducer of cell survival and metastasis (Agarwal, Brattain, & Chowdhury, 2013). Lin et al. also demonstrated that oral administration of cGMP to GC-C deficient mice reversed the accelerated epithelial proliferation that was observed before cGMP treatment. This evidence provides additional support to the hypothesis that the GC-C/cGMP pathway contributes to the suppression of intestinal tumorigenesis and carcinoma metastasis.

Cyclooxygenases

Cyclooxygenases (COX) are integral membrane glycoprotein enzymes that catalyze two steps in the conversion of arachidonic acid (AA) to pro-inflammatory prostaglandins (Andersen & Vogel, 2014). COX is somewhat unique in that it has two catalytic activities. COX first exerts its bis-oxygenase activity and converts arachidonic

acid to PGG_2. It then has a peroxidase activity, which results in the reduction of PGG_2 to PGH_2, which acts as the primary substrate for synthesis of specific prostaglandins (Minghetti, 2004).



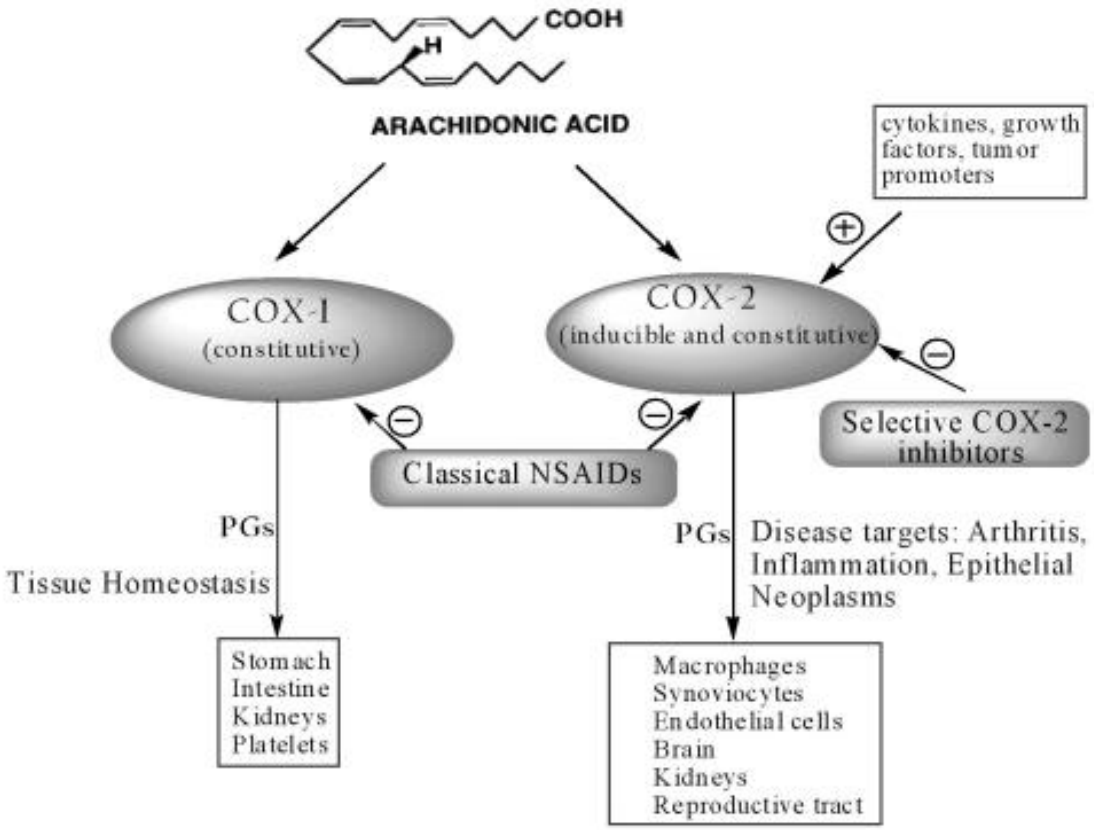
Adapted from (Zarghi & Arfaei, 2011).

Figure 6. Summary of Prostanoid Synthesis.

Cyclooxygenases catalyze two key reactions in biosynthesis of prostaglandins.

The two primary cyclooxygenase isoforms, COX-1 and COX-2, are coded by separate genes found respectively on chromosome 9 and 1. While COX-1 is

constitutively expressed and generally performs housekeeping functions, COX-2 is not usually expressed in normal tissues, but its expression can be rapidly induced by various stimuli including growth factors, pro-inflammatory molecules, and other cytokines (Sade, Tuncay, Cimen, Severcan, & Banerjee, 2012; Minghetti, 2004). The expression of COX-2 is regulated through a TATA box and several transcription factors with binding sites in the gene's promoter region (Minghetti, 2004). COX-1, on the other hand, lacks a TATA box and is thought of as a typical housekeeping protein. Because the two isoforms have functional domains that are mostly conserved, some COX inhibitors inhibit both forms. Several of the classical NSAIDs function by inhibiting both COX enzymes. Inhibitors specific to one isoform or the other have been discovered. COX-2-specific inhibitors were originally developed in hopes of preserving the housekeeping activity of COX-1, while suppressing the pro-inflammatory activity caused by COX-2 expression and activity (Andersen & Vogel, 2014).



Adapted from (Zarghi & Arfaei, 2011).

Figure 7. Roles of COX-1 and COX-2 and Differences between Classical NSAIDs and Selective COX-2 Inhibitors.

Inhibitors specific for COX-2 theoretically would preserve homeostatic cyclooxygenase activity via COX-1 while suppressing inflammation, cell growth, and tumor formation.

COX-2 expression has been associated with many disease states and has been shown to be induced by various mitogenic and inflammatory signals (Minghetti, 2004). In the gastrointestinal tract, COX-2 expression has been linked to the presence of certain bacteria, such as *Lactobacillus rhamnosos*, and may play a role in the immune response to environmental factors such as diet or the composition of the intestinal microbiome

(Korhonen, Kosonen, Korpela, & Moilanen, 2004). Inhibitors specific to the COX-2 isoform were originally developed to achieve anti-inflammatory and analgesic effects without the gastrointestinal toxicity caused by non-specific NSAIDs (Wallace & Devchand, 2005). However, COX-2 may have important roles in maintaining gastrointestinal homeostasis in response to environmental challenges. For instance, some of the prostaglandins produced following COX-2 activity appear to have important roles in strengthening mucosal defense by stimulating mucus secretion and maintaining blood flow to the mucosa. COX-2 inhibitors have been shown to delay healing of gastric ulcers (Wallace & Devchand, 2005), possibly as a result of restricting blood flow and causing disruption to the mucosal barrier in the gut.

While the mechanisms described above certainly play a role in the gastrointestinal toxicity observed following NSAID administration, they may also contribute to the role of COX-2 in CRC.

COX-2 and CRC

Constitutive COX-2 expression has been observed in many human tumors and appears to occur early on in the carcinogenic process, leading to tumor cell proliferation, angiogenesis, and eventual metastasis (Jendrossek, 2013). An increase in COX-2 expression has also been connected to poor prognosis in CRC patients (Lin, Lin, Lee, Liu, & Lee, 2013). In their study, Lin et al. examined COX-2 expression in human CRC tumors and adjacent normal mucosa. Most tumor specimens had very low levels of COX-2 compared to the surrounding normal tissue. However, when tumors had high COX-2 expression, the patients from which the specimens were derived had higher recurrence

rates and lower survival rates. As a result, COX-2-specific inhibitors have been explored as potential treatments for cancer.

COX-2 inhibition has been shown to sensitize tumor cells to chemotherapy or radiotherapy, and appears to induce apoptosis as well, potentially by inhibiting AKT, Survivin, and other molecules responsible for cell division and survival (Jendrossek, 2013). The exact mechanisms underlying the rationale for COX-2 inhibition in cancer treatment remain unclear, as COX-independent anti-neoplastic and pro-apoptotic effects following NSAID administration have been observed (Sade, Tuncay, Cimen, Severcan, & Banerjee, 2012). This suggests that there may be promiscuous activities from either the drugs themselves or their metabolites that are partially responsible for their effects. Nevertheless, strong data from *in vitro*, *in vivo*, and clinical studies support the potential for NSAIDs, particularly the COX-2 inhibitor celecoxib (Celebrex), in preventing and treating CRC (Lee et al., 2012; Pyrko et al., 2006; Nan et al., 2015; Jendrossek, 2013; Andersen & Vogel, 2014).

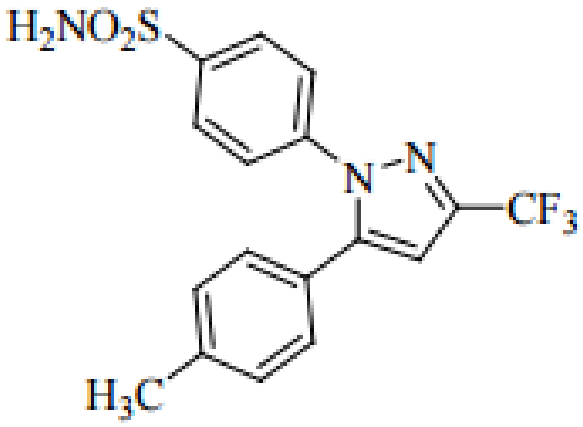
Celecoxib

Celecoxib was the first COX-2 selective inhibitor to be approved by any drug regulatory agency (McCormack, 2011). Other members of the COX-2 selective inhibitor class (i.e. –coxibs) include rofecoxib, valdecoxib, and etoricoxib (Zarghi & Arfaei, 2011), although celecoxib is the only currently approved –coxib. Celecoxib and other members of this class have been associated with adverse cardiovascular events. Rofecoxib was found to be associated with increased risk of heart attack and stroke, and

was withdrawn from the market in 2004. Valdecoxib was withdrawn the following year as well.

Celecoxib is currently approved by the United States Food and Drug Administration (FDA) for the treatment of several pain-related and inflammatory conditions, including osteoarthritis (OA), rheumatoid arthritis (RA), ankylosing spondylitis (AS), acute pain, and primary dysmenorrhea (GD Searle, 2011). The drug was also once approved by the EMA for the reduction of the number of adenomatous intestinal polyps in patients with FAP, but this approval has since been withdrawn. Nevertheless, coxibs have continued to be reputed for preventing development of adenomatous polyps. In fact, a significant reason for the withdrawal of rofecoxib was that an increase in cardiovascular risks was observed during a study that was assessing the drug's ability to prevent polyps (Zarghi & Arfaei, 2011). In one meta-analysis, celecoxib has not been found to significantly increase incidence of cardiovascular events over high doses of traditional NSAIDs (Kearney, Baigent, Godwin, Halls, Emberson, & Patrono, 2006), although this result is not consistent across other studies (McCormack, 2011). Celecoxib has been found in large safety studies to have significantly lower incidence of gastrointestinal (GI) complications compared to non-selective NSAIDs (McCormack, 2011).

Celecoxib was selected as the COX-2 inhibitor for use in the research presented in this thesis because of the decreased risk of GI toxicity and because celecoxib is the only COX-2 inhibitor approved by the FDA.



Celecoxib

Adapted from (Zarghi & Arfaei, 2011).

Figure 8. Chemical Structure of Celecoxib.

Rationale for Celecoxib/ST Combination in Treating CRC

Based on evidence supporting the independent use of GC-C agonists and celecoxib to prevent growth of CRC cells and induce tumor reduction, a combination of these two approaches may be an effective treatment approach in the CRC setting. However, Booth et al. (2015) recently showed that inhibition of PDE5, the molecule responsible for most cGMP degradation, by sildenafil potentiated the ability of celecoxib to induce apoptosis in human glioma cells. It is currently unclear whether similar effects would be obtained in CRC cells. Nevertheless, this study suggests that co-administration of celecoxib and GC-C agonists may lead to synergistic apoptotic and antiproliferative effects, as GC-C-induced cGMP accumulation may potentiate celecoxib's pro-apoptotic

activities in a manner similar to that observed following the inhibition of PDE5 by sildenafil. If a treatment with such effects could be translated to the clinic, it could provide physicians and patients with an important addition to the CRC therapeutic arsenal. However, the combination of celecoxib and a GC-C agonist may be best positioned in prophylactic regimens for patients with histories of adenomas or CRC or in patients with strong genetic predispositions for CRC, such as those with FAP, MAP, or HNPCC.

Chapter II

Materials and Methods

Cell Proliferation by Thymidine Incorporation

All cells used during the course of this research were T84 cells (ATCC P/N CCL-248), which are cultured from a human colorectal adenocarcinoma. T84 cells were cultured in DMEM/F-12 50/50 (Mediatech P/N 10-092-CV) supplemented with 1% L-Glutamine (VWR P/N 45000-676) and 5% Fetal Bovine Serum (FBS; VWR P/N 45001-106).

Cells were split at 20,000/well into four 96-well plates (Costar P/N 3610) using the medium described above and were incubated at 37°C for twenty-four hours. Cells were then starved of FBS to synchronize cell cycles by removing the complete media, washing the cells with 100µL of DMEM/F-12, and replacing the medium with 100µL of DMEM/F-12. Cells were starved for twenty-four hours.

After synchronization, the media were removed and replaced with 80µL of DMEM/F-12, 10µL of treatment (treatments described in **Table 1**), and 10µL of FBS (rows B-H) or 10 µL of DMEM/F-12 (row A). All treatments contained 0.5% dimethyl sulfoxide (DMSO) because DMSO is necessary for celecoxib solubility. Camptothecin (CPT; Tocris P/N 1100) was used as a positive anti-proliferation control. 1 µM ST (PolyPeptide Laboratories P/N 0603-156) was used both in combination with varying

doses of celecoxib and alone. Two plates were incubated at 37°C for eighteen hours and two more were incubated for forty-two hours. After incubation, 0.2μCi of [^{3}H]-thymidine (American Radiolabeled Chemicals P/N ART 0178A) was added to each well. The plates were then incubated for an additional six hours to allow the radiolabeled thymidine to be incorporated into the cells' DNA.

Row	Columns 1-3 (celecoxib alone)	Columns 4-6 (CPT)	Columns 7-9 (celecoxib + **1 μM ST**)	Columns 10-12 (ST alone)
A	Vehicle (no FBS)			
B	Vehicle			
C	0.125 μM	0.00001 μM	0.125 μM + ST	0.00001 μM
D	1.25 μM	0.0001 μM	1.25 μM + ST	0.0001 μM
E	6.25 μM	0.001 μM	6.25 μM + ST	0.001 μM
F	12.5 μM	0.01 μM	12.5 μM + ST	0.01 μM
G	25 μM	0.1 μM	25 μM + ST	0.1 μM
H	50 μM	1 μM	50 μM + ST	1 μM

Table 1. Treatments in Cell Proliferation Assay (Thymidine Incorporation).

After incubation, the medium was removed and 50μL of trypsin was added to each well. Once the cells were visibly detached from the plates, they were transferred onto a filter plate (Millipore FB P/N MADVNOB) that had been pre-washed with 150 μL of water and connected to an aspirator. Each filter plate was washed with 150 μL of water 6 times. When all liquid was aspirated after the last wash, each filter was removed and allowed to dry for 60 minutes in a 50ºC oven. After drying, filters were attached to 96 well plates for counting. 80 μL of microscint (UltraGold MV, PerkinElmer P/N 6013159)

was added to each well and the amount of radioactivity was counted using a MicroBeta liquid scintillation counter.

COX-2 Activity Assay

The COX-2 activity assay was conducted using the COX Activity Assay Kit (Cayman Chemical P/N 760151). T84 cells were cultured in DMEM/F-12 50/50 supplemented with 1% L-Glutamine and 5% FBS and were split into two 24-well plates. Because 0.5% DMSO was used in the proliferation assay, the same concentration was used in the enzyme activity assays in each well. Cells were treated with vehicle (0.5% DMSO), 1μM ST, or the combination of 50μM celecoxib and 1μM ST. Cells were subsequently incubated at 37° for 24 hours.

Following treatment, cells were scraped from the plates using a rubber policeman and were harvested into 500 μL Eppendorf tubes. The tubes were centrifuged at 4°C for 10 minutes at 1,000xG. The supernatant was extracted and pellets were sonicated in a buffer of 0.1M Tris-HCl pH 7.8 with 1mM EDTA. The cell fragments and buffer were then centrifuged at 4°C for 15 minutes at 10,000 x *g*. Supernatant was extracted and stored at -80°C.

After samples were thawed, 150 μL of each sample was transferred to a 500 μL microfuge tube. The tubes were centrifuged at 8,000 x *g* for one minute. For the COX standard wells (n=4; "Std" in Figure 14), 150 μL assay buffer (100 mM Tris-HCl, pH 8.0), 10 μL Heme, and 10 μL of the provided standard was added to a 96-well plate. The background wells (n=4) were prepared using 120 μL assay buffer, 10 μL Heme, and 40

µL distilled water. Sample wells taken from each of the treatment groups (vehicle, ST alone, ST/celecoxib combination; n=16 each) were prepared using 120 µL assay buffer, 10 µL Heme, and 40 µL of sample from the centrifuged microfuge tubes. The plate was then shaken gently for a few seconds and was incubated for 5 minutes at room temperature.

After 5 minutes, 20 µL Colorimetric Substrate was added to each well. The reactions were then initiated by adding 20 µL arachidonic acid solution to each well. The plate was again shaken briefly and allowed to incubate for 5 minutes at room temperature. Following incubation, absorbance at 590 nm was read using a plate reader.

Average absorbance was calculated for each treatment group and the average background value was subtracted from each treatment average. These corrected values are the corrected absorbances (ΔA_{590}). The corrected absorbances were subsequently inserted into the following formula to calculate total COX activity.

$$\text{Total COX Activity} = \frac{\Delta A_{590}/5 \text{ min.}}{0.00826\ \mu M^{-1}} \times \frac{0.21 \text{ ml}}{0.04 \text{ ml}} \div 2^* = \text{nmol/min/ml (U/ml)}$$

Adapted from COX Activity Assay Kit.

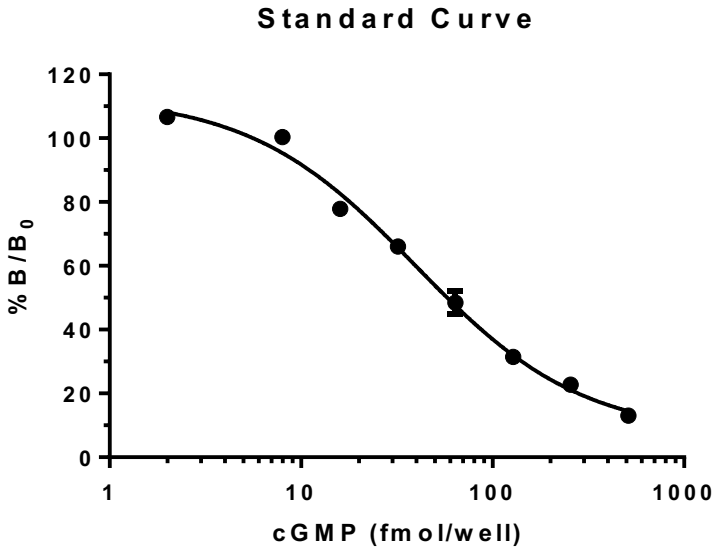
Figure 9. Equation for Calculating Total COX Activity.

cGMP Accumulation Assay

GC-C activity, measured by accumulation of cGMP, was assessed using a modification of the method described by Busby et al. (2010). T84 cells were cultured in a 24-well plate in DMEM/F12 supplemented with 2.5 mM glutamine and 5% FBS. Total medium per well was 1mL. 30 minutes before treatment, the medium was removed and replaced with a medium containing 1 mM isobutylmethylxantine (IBMX). Plates were incubated for 30 minutes at 37°C. The medium was then removed and vehicle wells (n=8) were filled with 1 mL DMEM/F12 supplemented with 2.5 mM glutamine, 5% FBS, and 1 mM IBMX. Wells assigned to the ST treatment group were filled with 1 mL DMEM/F12 supplemented with 2.5 mM glutamine, 5% FBS, 1 mM IBMX, and 1 μM ST (n=8). Wells assigned to the combination treatment group were filled with the same medium as the other groups as well as 50 μM celecoxib. The plate was then incubated for 30 minutes in a 37ºC/5% CO_2 incubator. After the 30 minutes of exposure, the media from the wells were aspirated and the cells were lysed in 1 mL of cold 0.1 M HCl and underwent one cycle of freezing at -80ºC and thawing. Cell lysates were then transferred to 1.5 mL microfuge tubes and were cleared of cell debris by centrifugation at 15,000 X g at 4ºC. Protein concentration in the lysates was measured by the method of Bradford (1976).

The concentration of cGMP in the T84 cell lysates was measured using a cGMP enzyme immunoassay kit (GE Healthcare Amersham cGMP Enzymeimmunoassay Biotrack System). 20 μL cell lysate from the vehicle treated cells was added to 4.5 μL 1M Tris-HCl pH 8 and 175.5 μL assay buffer (50 mM sodium acetate pH 6 containing 0.002% BSA and 0.01% preservative). 2 μL cell lysate from ST and ST/celecoxib treated

cells was added to 1.25 µL 1M Tris-HCl pH 8 and 196.75 µL assay buffer. cGMP in standards (2-512 fmol, prepared fresh in 200 µL assay buffer) and diluted cell lysates were acetylated with 20 µL acetylation reagent (1 part acetic anhydride, 2 parts triethanolamine). A zero standard was also included that did not contain any cGMP. Next, 50 µL of acetylated standards and samples were added to each well of a 96-well microplate, coated with donkey anti-rabbit IgG, containing 100 µL of diluted anti-cGMP serum. Two wells contained only 150 µL assay buffer and served as non-specific binding controls. After the plate was incubated for two hours at 4ºC, 100 µL of diluted cGMP conjugated with horse radish peroxidase (HRP) was added to each well and the plate was incubated for another hour at 4ºC. The contents from all wells were aspirated and each well was washed 4 times with 250 µL wash buffer (0.01 M phosphate buffer pH 7.5 containing 0.05% Tween 20). After the last wash was removed and the plate blotted, 200 µL HRP substrate (3,3',5'5'-tetramethylbenzidine/hydrogen peroxide) was added and the blue color was allowed to develop for 20 minutes at room temperature. Color development was stopped by the addition of 100 µL of 1 M sulphuric acid. The optical density was read at 450 nm. For each sample, net optical density was determined by subtracting the optical density of the non-specific binding wells from the raw optical density of each sample. The $\%B/B_0$ was calculated by dividing the net optical density of each sample by the net optical density of the zero standard (B_0) x 100. A standard curve (Figure 10) was generated by plotting the $\%B/B_0$ as a function of log cGMP concentration. cGMP in the cell lysates was calculated using the standard curve.



Adapted from GE Healthcare Amersham cGMP Enzymeimmunoassay Biotrack System.

Figure 10. Example of cGMP Standard Curve.

Western Blot Experiments

After treatment (same treatments as used in COX and GC-C activity assays), the medium was aspirated and cells were washed in PBS once. Cells were subsequently lysed in PBS-0.2% Triton X-100 supplemented with protease and phosphatase inhibitors. Protein concentration in the lysates was assessed using the method described by Bradford (1976), using gamma globulin as the standard.

SDS-polyacrylamide gel electrophoresis and immunoblots were conducted using a modification of the method described by Kessler et al. (1995). Purified human COX-2 (1mg, Sigma P/N C0858) and cell lysate proteins (4µg, 12µg, 40µg, and 104µg) were mixed with gel loading buffer, and were heated for 10 minutes at 70°C. Proteins were then separated in SDS-4-12% (NUPAGE Precast Mini Bis-Tris) using a constant voltage of 200V at room temperature for 40 minutes. After separation, the gels were soaked in transfer buffer (25mM Tris/192 mM glycine and 20% methanol) for 5 minutes, and proteins were transferred to polyvinylidene difluoride (PVDF) according to the method described by Towbin et al. (1979). Transfers were run overnight using a constant current of 10mA.

After the proteins were transferred, the membranes were soaked in water for 5 minutes and Super Block PBS buffer (Thermo Fisher P/N 37515) containing 0.05% Tween-20 was applied for one hour at room temperature to block nonspecific protein binding sites on the membranes. Blotted proteins were treated overnight at 4°C with diluted anti-COX-2 antibody (Cell Signaling P/N 4842, diluted 1:1,000) or with diluted anti-Caspase-3 antibody (Santa Cruz Biotechnology P/N sc-7148 , diluted 1:5000) After

being washed 4 times in wash buffer (10mM Tris-HCL, ph 8, 150mM NaCl, 0.05% Tween 20) for 5 minutes per wash, the blots were treated with horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody for 1 hour at room temperature. The blots were again washed 4 times in wash buffer. Immunoreactive peptides were visualized by reaction of the blots with the HRP chemiluminescent substrate system (Cell Signaling Technology, SignalFire™ ECL Reagent) and detection in X-ray film.

Chapter III

Results

Results of Thymidine Incorporation Assays

The positive control CPT (DNA topoisomerase inhibitor) dose-dependently inhibited thymidine incorporation (cell division) in T84 cells, validating the anti-proliferation assay. Celecoxib was found to dose-dependently inhibit thymidine incorporation/cell proliferation. The combination of ST and celecoxib, however, failed to further reduce thymidine incorporation in comparison to celecoxib alone. ST alone did not affect thymidine incorporation at any dose. These results were consistent between the 24 and 48 hour time points at which tritium radiation was measured (Figure 11 and Figure 12).

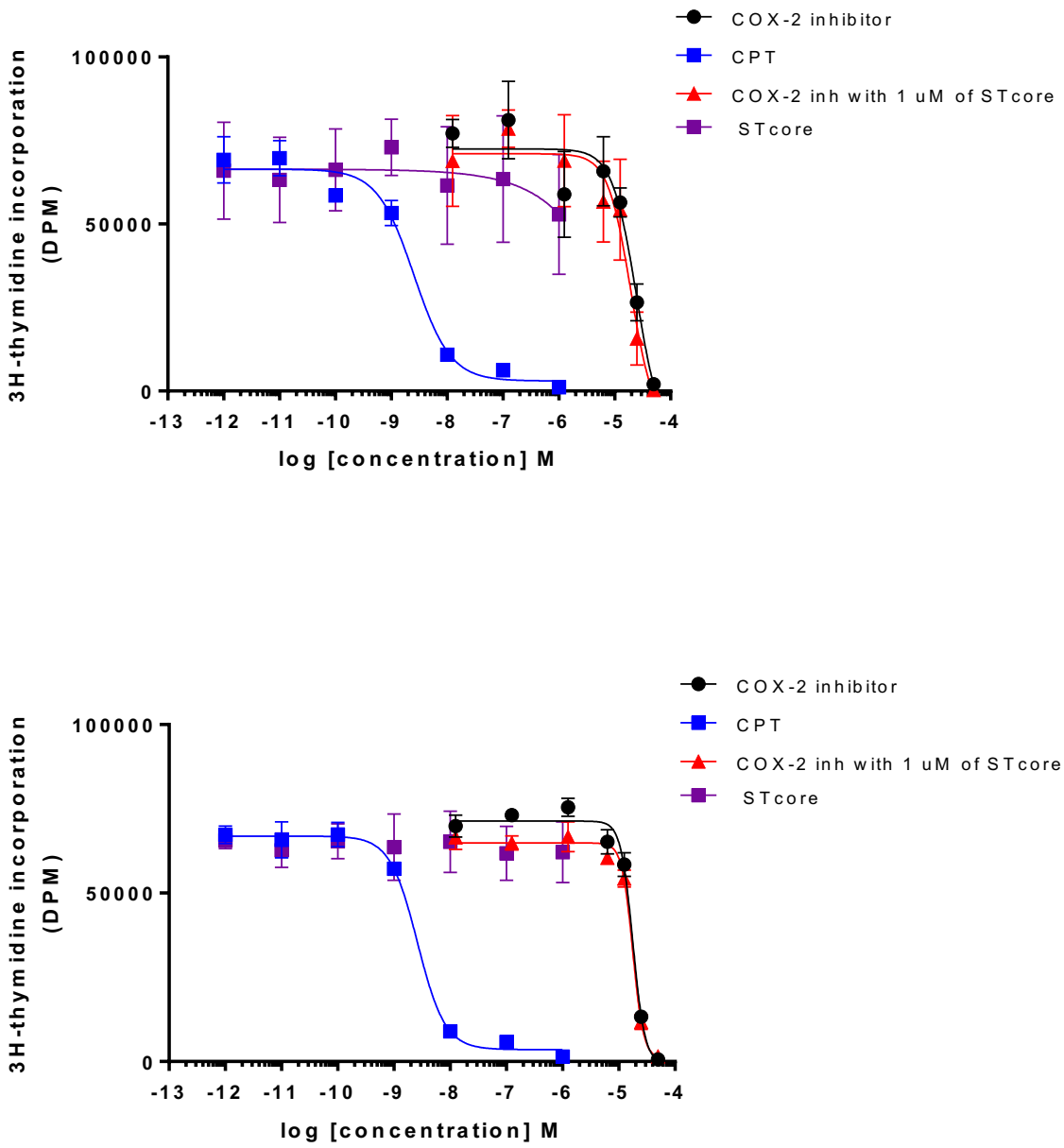


Figure 11. Thymidine Incorporation Results from 2 Separate 96-well Plates over 24 Hours.

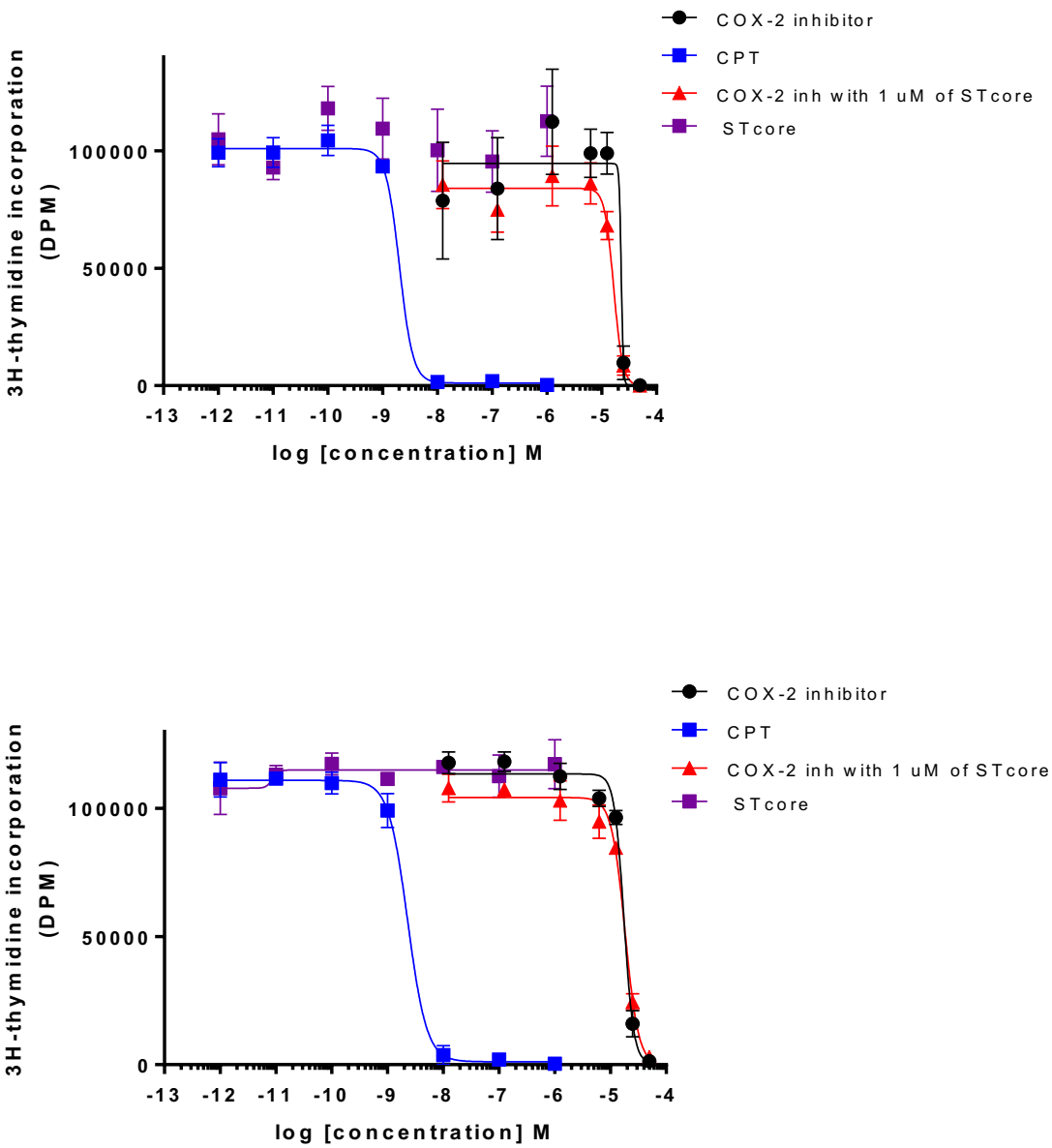
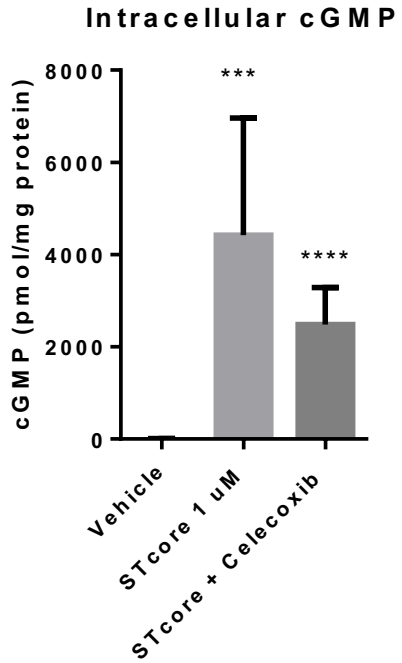


Figure 12. Thymidine Incorporation Results from 2 Separate 96-well Plates over 48 Hours.

Results of cGMP Accumulation Assay

The activity of GC-C, measured by accumulation of intracellular cGMP, was found to be significantly increased in cells treated with ST and in cells treated with the combination of ST and celecoxib in comparison to cells treated with vehicle alone (Figure 13). In cells treated with both ST and celecoxib, there was an apparent reduction in cGMP accumulation compared to cells treated with only ST. However, this difference was not statistically significant, which suggests that celecoxib does not interfere with GC-C activity or with ST's ability to agonize such activity.



$***P < 0.001$, $****P < 0.0001$ (compared to vehicle)

Figure 13. Levels of Intracellular cGMP in T84 Cells Measured after Treatment.

Results of COX Activity Assay

There was no difference in COX activity across the treatment groups (Figure 14). While the assay measured total COX activity and not COX-2 activity specifically, it is presumed that any change in COX activity in this experiment would be the result of changes to the COX-2 isoform due to the well-recognized specificity of celecoxib for COX-2 and not COX-1.

The results from this experiment provide further support for the hypothesis that COX-2 is not expressed in T84 cells and that the anti-proliferative activity of celecoxib occurs through a mechanism independent of COX-2.

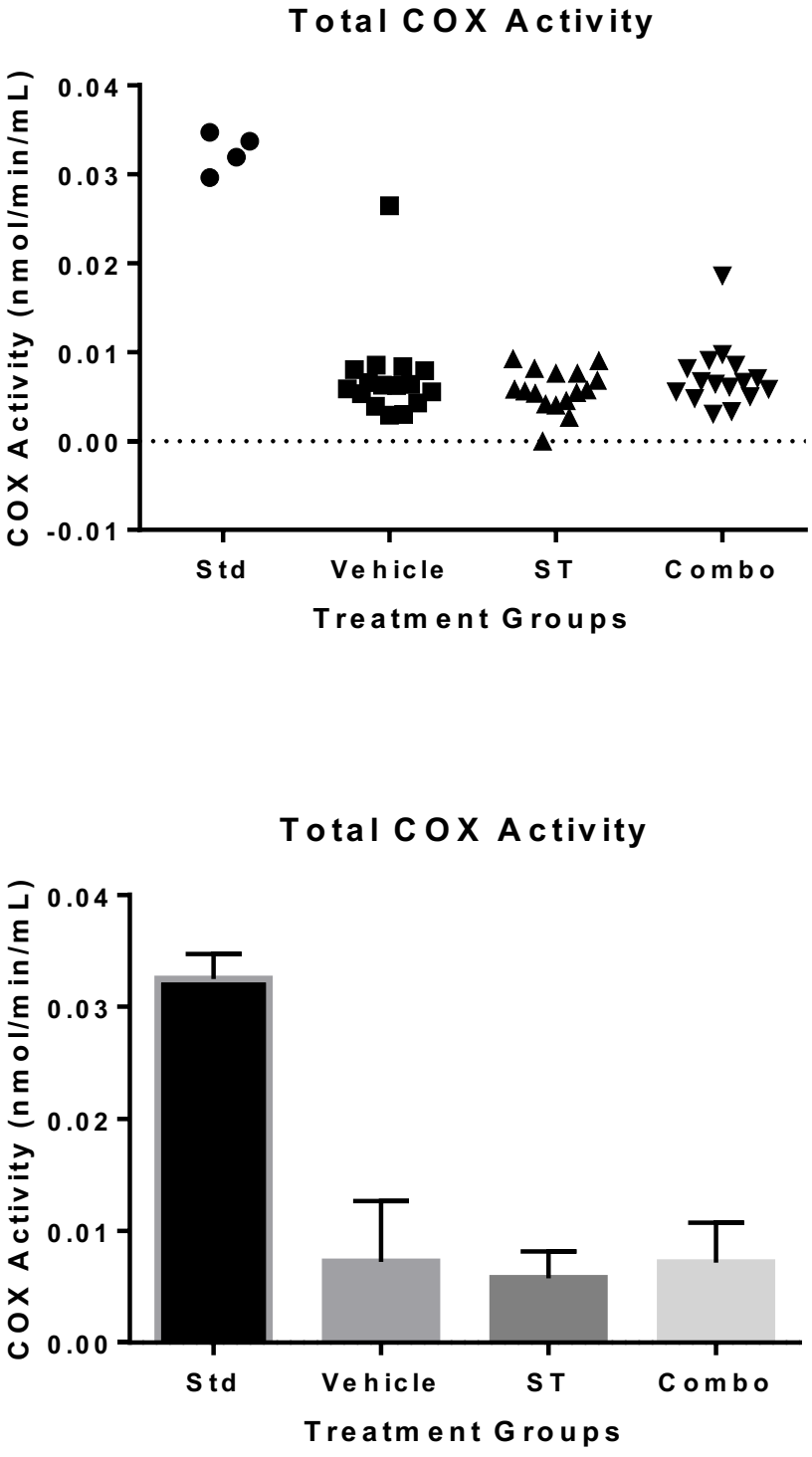


Figure 14. **COX Activity Measured in T84 Cells after Treatment.**

Results of COX-2 Western Blot Experiment

A western blot analysis using T84 cell lysates was used to confirm that COX-2 protein is not detectable in these cells (Figure 15). Recombinant human COX-2 was used as a positive control for the blot. In addition to not being detected in the untreated T84 cells, COX-2 was not detected in any of the treatment groups. There appears to have been cross-reaction between the COX-2 antibody and another protein (35 kDa) in the T84 lysates. This cross-reaction may be an anomaly, or, as described in the discussion section, below, may be an interesting lead into identifying the COX-2-independent anti-proliferative mechanism of celecoxib.

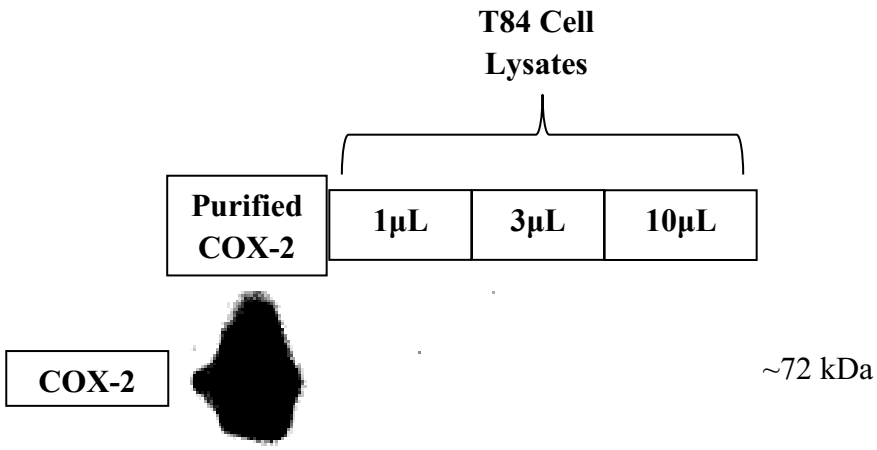


Figure 15. Results of Western Blot Experiment for COX-2 in T84 Cells.

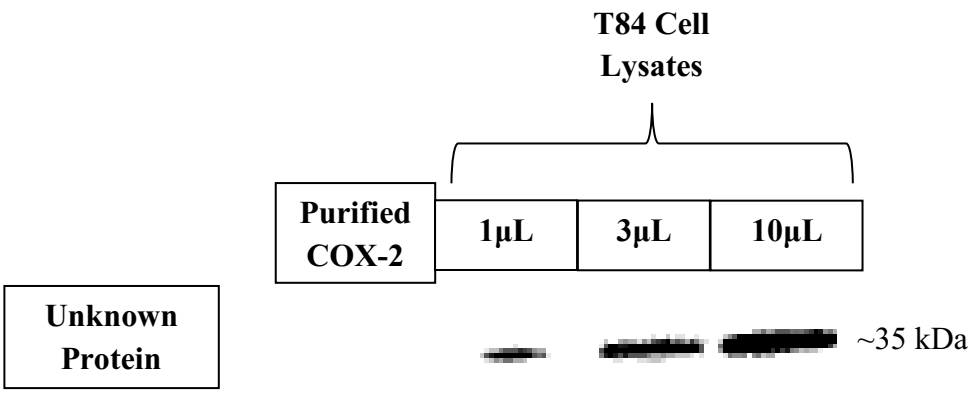


Figure 16. Unknown Protein Found to Cross-react with Anti-COX-2 Antibody.

Results of Caspase-3 Western Blot Experiment

Caspase-3 was not detected in any of the treatment groups. Interestingly, this marker of cell death was not detected in LPS-treated T84 lysates either, which is surprising because LPS is expected to cause cell death, and therefore lead to detectable Caspase-3 levels.

Chapter IV

Discussion

The results of these experiments do not support the hypothesis that GC-C agonism and COX-2 inhibition synergistically reduce tumor cell proliferation. Celecoxib was found to reduce proliferation of T84 cells in a dose-dependent manner. The potency of this reduction in proliferation was several orders of magnitude lower than that of the positive control, camptothecin. ST, the potent GC-C agonist used in this research, did not significantly reduce proliferation when used as a monotherapy, and did not potentiate the effects of celecoxib when the two drugs were used in combination.

The thymidine incorporation assay results appear to contradict the work previously published by Pitari et al. (2001). Unpublished research by the laboratory in which this thesis was conducted has also been unable to find an anti-proliferative effect of GC-C agonists in T84 cells. While ST did not significantly potentiate celecoxib's anti-proliferative effects in this study, this result may be attributed to celecoxib's comparatively potent activity, which may have made it difficult to detect whether there was a subtle reduction in proliferation after ST administration. In fact, according to Figures 11 and 12, it appears that there may actually be a slight reduction in proliferation even though the difference was not statistically significant.

Although celecoxib was able to prevent proliferation, this effect appears to be independent of the COX-2 enzyme, as COX-2 protein was not found in T84 cells. This is consistent with previous research demonstrating that COX-2 is not expressed under normal physiological conditions (Korhonen, Kosonen, Korpela, & Moilanen, 2004) and with other work showing that celecoxib's antiproliferative effect is independent of COX-2 in other types of cancer as well as in CRC (Sade et al., 2011; Gaullouet et al., 2014). Furthermore, a reduction in arachidonic acid was not observed in treated cells. This supports the finding that COX-2 is not expressed in these cells, as celecoxib is known to be a potent inhibitor of COX-2 and not of COX-1.

The presence of Caspase-3 was used to assess whether any of the treatments had a cytotoxic effect. The highest dose of celecoxib was used in the Western blot experiment because it was assumed that any effects on enzyme activity would be best observed using the highest dose. It has previously been demonstrated that Caspase-3 is activated and detectable in T84 cells following incubation with *Clostridium difficile* Toxin A (Carneiro et al., 2006). In their experiment, Caspase-3 was not detected by Western analysis until 18 hours of incubation, and levels appeared to peak at 48 hours. As expected, ST activation of GC-C had no cytotoxic effects, as measured by Caspase-3 induction. ST did not induce apoptosis when combined with celecoxib. LPS was unable to produce detectable Caspase-3 even after 24 hours of incubation. It is possible that either an increase in the concentration of LPS or the incubation time may be required to cause a detectable increase in the apoptotic protein.

At first glance, these results appear to conflict somewhat with the data generated by Booth et al. (2015) in which the researchers found that celecoxib had cytotoxic effects and that these effects were potentiated by inhibiting the PDE5, which results in an accumulation of cGMP. In the research presented in this thesis, a high dose of celecoxib was not found to be cytotoxic as shown by a lack of detectable protein in a caspase-3 western analysis. This result is inconsistent with previous additional previous work from Gallouet et al. (2014), suggesting perhaps that the occurrence of cytotoxic activity of celecoxib may be variable based on cell type.

The inability of GC-C agonism to reduce proliferation was not entirely unexpected after reading the work of Li et al. (2007). In this paper, the authors suggest that inhibiting hyperproliferation was the primary mechanism by which GC-C prevents tumor development and growth in the small intestine. In the large intestine, however, the authors found that reduction in tumor burden was instead associated with reduced genomic abnormalities. Cell proliferation in the large intestine appeared to be unaffected. As a result, the failure of ST to reduce proliferation in T84 cells, which are, of course, cancerous cells derived from the colon and not the small intestine, is not inconsistent with previous work conducted *in vivo*.

Limitations

One of the primary limitations of these experiments is that they were all conducted in T84 cells. While this is a commonly used cell line in research of gastrointestinal function and disorders, it is, of course, derived from a single

adenocarcinoma from a single patient. It could therefore differ significantly in terms of DNA and protein expression and other factors from other human CRC-derived cell lines and ultimately from the CRC found in most patients. More robust results could be obtained by conducting the same experiments in multiple cell lines, such as in HCT 116 cells.

While a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay would have been the preferred method of assessing whether celecoxib and the combination of ST and celecoxib induced apoptosis, this was not practical during the course of the research described here for lab-specific reasons. The Caspase-3 Western protocol had been used successfully in the lab in which this research was conducted and was deemed to be a suitable replacement to assess whether the treatments were cytotoxic. Interestingly and unexpectedly, Caspase-3 was not detected in LPS-treated T84 cells as described above. With more time and resources, it would be worthwhile to find another method to assess cytotoxicity. For instance, in the research conducted by Carneiro et al. (2006), Caspase-8 and Caspase-9 were detected prior to Caspase-3. Perhaps these proteins may be more easily detected in T84 cells following treatment with LPS or ST/celecoxib.

Future Directions

As described above, the role of GC-C in preventing tumor growth and development may be different in the colon versus the small intestine. It would be interesting to perform a similar experiment assessing changes in genomic integrity, rather

than proliferation, to evaluate whether activation of GC-C may actually prevent carcinogenesis at a genomic level. For example, looking at markers of DNA repair or chromosomal stability may provide additional insight into the mechanism by which GC-C controls CRC development, if, in fact, it does. In addition, it may be worthwhile to test whether a GC-C agonist can reduce proliferation in adenocarcinomas derived from the small intestine.

An obvious progression from this research would be to assess whether celecoxib-like compounds with no activity on COX-2 have the same effect on proliferation. If the anti-proliferative effects were found to be the same, this would provide additional confirmation that celecoxib inhibits cell proliferation through a pathway independent of COX-2.

One of the more interesting results from this research was the finding of a cross-reactant protein (at ~35 kDa) to the anti-COX-2 antibody used in the Western experiment. While this finding could easily be anomalous, resulting simply from random cross-reactivity, it could also be an intriguing starting point for further research into the mechanism by which celecoxib exerts its anti-proliferative effects. If the anti-COX-2 antibody is able to bind to this mystery protein, it follows that the protein may share a binding region with COX-2, which could be the location at which celecoxib acts in both proteins.

It may be worthwhile to investigate the identity of this unknown protein and assess whether celecoxib actually binds directly to it. If celecoxib is found to bind to the unknown protein, a suitable next step might be to evaluate the function of this protein to

see if it is involved in any way in the control of the cell cycle or in any apoptotic pathways. If this is found to be the case, it may explain celecoxib's COX-2-independent effects.

Other Combinations with GC-C Agonists

Although agonism of the GC-C pathway with ST did not inhibit cell proliferation in this study either alone or in combination with celecoxib, it is possible that additional drug combinations may result in enhanced activity against CRC.

Activation of the tyrosine kinase c-src has been strongly associated with CRC and appears to be an indicator of poor prognosis in patients with increased expression and activity (Basu, Bhandari, Natarajan, & Visweswariah, 2009). Interestingly, GC-C has been identified as a substrate of c-src phosphorylation (Basu et al., 2009; Singh, 2003). This phosphorylation has an inhibitory effect on GC-C, leading to a reduction in cGMP following binding of guanylin, uroguanylin, ST, or another GC-C agonist when compared to the un-phosphorylated enzyme. Inhibition of c-src using dasatinib, which is approved for treatment of several cancers and has been shown to be efficacious in killing human CRC cells (Kopetz et al., 2009), may therefore potentiate the activity of a GC-C agonist in an environment in which c-src is active. This may simply lead to an increase in the effects typically seen upon GC-C agonism (increased motility and decreased pain). However, if GC-C is in fact partly responsible for cell cycle regulation (though this effect was not seen in the research presented in this thesis), the potentiation of its activity may result in decreased tumor growth.

Basu et al. (2009) further demonstrate that phosphorylation of GC-C leads to additional activation of c-src. It follows that activation of GC-C above levels typically induced in the gut by the endogenous hormones guanylin and uroguanylin may lead to decreased c-src activation, although this depends entirely on whether decreased cGMP or simply the presence of the additional phosphate group on GC-C is responsible for the enhanced c-src activation.

Other Combinations with COX-2 Inhibitors

Although the effects on proliferation seen after celecoxib administration appear to be independent of COX-2, the prostanoid biosynthesis pathway mediated by the cyclooxygenases may be an interesting target for other drug combinations with potential synergistic effects. Such combinations may be ideally suited for inflammatory conditions, rather than cancer.

One combination that may be worth investigating in gastrointestinal inflammatory disorders could include a COX-2 inhibitor and a fatty acid amide hydrolase (FAAH) inhibitor. The FAAH enzyme mediates the hydrolysis of anandamide (AEA) and other endogenous fatty acid amides that act as cannabinoids (i.e. they act on cannabinoid receptors) (Romero, Hillard, Calero, & Rabano, 2002). Because anandamide hydrolysis produces arachidonic acid, the primary substrate for cyclooxygenases, dual FAAH and COX-2 inhibition may lead to synergistic anti-inflammatory effects. One important outcome of this potential synergism is that co-administration of a FAAH inhibitor may allow for a reduction in the dose of a COX-2 inhibitor. As described earlier in this paper,

even though COX-2 inhibitors are effective NSAIDs, they are also associated with serious cardiovascular concerns. As a result, if a combination product could produce the same anti-inflammatory effects without needing high doses of the COX-2 inhibitor, it may serve as a helpful addition to physicians' treatment arsenal. Of course, the safety and tolerability of each drug involved in the combination and the combination itself would need to be rigorously assessed in addition to efficacy.

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Appendix

Definition of Terms

“Adenoma”: A benign tumor formed from glandular structures in epithelial tissue

“Agonist”: A substance that binds to and activates a receptor to stimulate activity by the receptor

“Apoptosis”: The process of programmed cell death that occurs in multicellular organisms

“Benign”: Lacking the ability to invade neighboring tissue or metastasize; Not cancerous

"cAMP": Cyclic Adenosine Monophosphate. A second messenger derived from adenosine triphosphate (ATP) and used for intracellular signal transduction.

“Carcinoma”/“adenocarcinoma”: A malignant tumor derived from epithelial cells

"cGMP": Cyclic Guanosine Monophosphate. A second messenger derived from guanosine triphosphate (GTP) and used for intracellular signal transduction

“COX”: Cyclooxygenase. An enzyme that catalyzes the formation of pro-inflammatory compounds called prostaglandins from arachidonic acid.

“Endogenous”: Originating from within an organism

“Epigenetic”: Relating to or arising from nongenetic influences on gene expression

“Epithelial tissue”: One of the four basic types of animal tissue. Epithelial tissue lines cavities and surfaces of blood vessels and organs throughout the body.

"GC-C": Guanylate Cyclase C. An enzyme found in the luminal intestinal epithelial that catalyzes the formation of cGMP.

"Guanylin": A peptide secreted by colonic goblet cells that acts as an agonist of GC-C.

“Inhibitor”: A substance that binds to an enzyme and decreases its activity.

“Lumen”: The inside space of a tubular structure (e.g. intestine).

“Malignant”: Tending to invade normal tissue or to recur after removal; Cancerous.

“Metastasis”: The spread of cancer from one organ or part to another not directly connected organ or part.

“Neoplasia”: Abnormal growth of tissue.

“Paracrine”: A form of cell to cell communication in which a cell produces a signal to induce changes in nearby cells.

“Pathogenesis”: The manner of development of a disease.

“Polyp”: Abnormal growth of tissue projecting from a mucous membrane.

“Proliferation”: Rapid reproduction of cells.

"Uroguanylin": A peptide secreted by enterochromaffin cells that acts as an agonist of GC-C.

“Second messenger”: A molecule that relays signals received at receptors on the cell surface to target other molecules in the cell.

"ST": Heat-Stable Enterotoxin. Bacterial derived secretory peptides that act as potent agonists of GC-C.

“Synergism”: Interaction of discrete agents such that the total effect is greater than the sum of individual effects.