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Chlor-Amidine, A Novel PAD Inhibitor, As An Effective Drug For The Treatment Of Ulcerative Colitis And Prevention Of Colorectal Cancer

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CHLOR-AMIDINE, A NOVEL PAD INHIBITOR, AS AN EFFECTIVE DRUG FOR THE TREATMENT OF ULCERATIVE COLITIS AND PREVENTION OF COLORECTAL CANCER

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DEDICATION

This dissertation is dedicated to my loving husband, family, and friends who have supported me throughout my graduate work.

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ABSTRACT

Ulcerative colitis (UC) is a chronic inflammatory bowel disease that affects the quality of life of millions of patients worldwide. This disease is associated with inflammation and ulceration of the colonic epithelium, leading to an increased risk for the development of UC-associated colorectal cancer (CRC). Current UC medications are designed to manage the symptoms and induce remission; however, several challenges are faced with current treatment options. 5-aminosalicyclic acid and corticosteroids have few side effects, but have limited efficacy and often the disease becomes refractory. Biologics are introduced after initial treatments fail, but serious side effects are often associated with these medications. In the hope of developing a safe and effective UC therapeutic, we are testing a small molecule inhibitor of Protein Arginine Deiminases (PADs), which are implicated in many inflammatory diseases, like Alzheimer's disease, Multiple Sclerosis, Rheumatoid Arthritis, and UC.

PADs catalyze the conversion of peptidyl-Arginine to peptidyl-Citrulline through a process termed 'citrullination'. Chlor-amidine (Cl-amidine) is designed to irreversibly inhibit PADs through covalent modification at the active site of the enzymes. Our initial studies have determined that Cl-amidine is generally nontoxic in cell and animal models. Next, we tested the efficacy of Cl-amidine in the HCT116 colon cancer cell line and the Azoxymethane (AOM)/Dextran Sulfate Sodium (DSS) murine model of UC. We showed that Cl-amidine effectively reduced inflammation, oxidative stress produced by activated

leukocytes, and DNA damage. Based on these findings, we hypothesized that Cl-amidine could prevent the progression to UC-associated CRC. In vitro studies indicated that Cl-amidine may act as a tumor suppressor by upregulating miR-16, a putative tumor suppressor miRNA with cell cycle targets, and causing G1 cell cycle arrest. Our results showed that in the AOM/DSS murine model of UC-associated CRC, Cl-amidine significantly inhibited tumorigenesis, further supporting our hypothesis.

For a mechanistic study of Cl-amidine, we explored the idea that Cl-amidine may be preventing the citrullination and/or hyperactivity of DNA methyltransferases (DNMTs). Hypermethylation is reported to cause methylation-mediated gene silencing; therefore, we postulated that PADs may be citrullinating DNMTs and causing hypermethylation of genes regulating tumor suppressor miRNAs. We verified that DNMTs can be citrullinated and that the inhibition of DNMTs or PADs restores miR-16 levels to comparable levels. Cl-amidine also reduced the methylation of the miR-16 promoter as effectively as DNMT inhibition. Ultimately, the objective of our research is to provide evidence of nontoxic and more successful treatment options for UC.

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List of Abbreviations

5-ASA ..... 5-aminosalicyclic acid

5-Aza 5-Azacytidine

5-mC 5-methylcytosine

Ab ..... Antibody

AIF ..... Apoptosis-inducing factor

AOM ..... Azoxymethane

Arg ..... Arginine

BAA ..... Benzoyl-arginine amide

Bax ..... Bcl-2 associated X protein

bp ..... Base pairs

Cdks ..... Cyclin-dependent kinases

cDNA ..... Complementary deoxyribonucleic acid

Cit ..... Citrulline

Cl-amidine ..... N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide or Chlor-amidine

CLL ..... Chronic lymphocytic leukemia

CNS ..... Central nervous system

Cox-2 ..... Cyclooxygenase-2

CpG ..... Cytosine phosphodiester guanine

CRC ..... Colorectal cancer

Ct ..... Comparative threshold cycle

Cys ..... Cysteine

DAI ..... Disease activity index

DLEU2 ..... Deleted in leukemia gene 2

DMEM ..... Dulbecco's Modified Eagle Medium

DNA ..... Deoxyribonucleic acid

DNMTs ..... DNA methyltransferases

DSBs ..... Double-strand breaks

DSS ..... Dextran sulfate sodium

EAE ..... Experimental autoimmune encephalomyelitis

EC_{50} ..... 50% of maximal effective concentration

EDTA ..... Ethylenediaminetetraacetic acid

ELISA ..... Enzyme-linked immunosorbent assay

EMT ..... Epithelial-to-mesenchymal transition

FBS ..... Fetal bovine serum

FC ..... Fold change

G1, G2 phase ..... Gap 1, Gap 2 phase

GADD45 ..... Growth arrest and DNA damage protein 45

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

gDNA ..... Genomic deoxyribonucleic acid

GFAP ..... Glial fibrillary acidic protein

GPx1 ..... Glutathione peroxidase 1

GRIP1 ..... Glucocorticoid receptor-interacting protein 1

GSK3β ..... Glycogen synthase kinase 3β

H&E ..... Hematoxylin and Eosin

HDAC ..... Histone deacetylase

HED ..... Human equivalent dose

H_2O_2 ..... Hydrogen peroxide

IBD ..... Inflammatory bowel disease

IFN-γ ..... Interferon-gamma

IHC ..... Immunohistochemistry

ING4 ..... Inhibitor of growth 4

iNOS ..... Inducible nitric oxide synthase

i.p. ..... Intraperitoneal

IP ..... Immunoprecipitation

kDa ..... Kilodalton

KO ..... Knockout

MBP ..... Myelin basic protein

MDM2 ..... Mouse double minute 2

miR/miRNA ..... MicroRNA

MLH1 ..... MutL homolog 1

M phase ..... Mitosis phase

mRNA ..... Messenger ribonucleic acid

MS ..... Multiple sclerosis

NET ..... Neutrophil extracellular trap

NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NLS ..... Nuclear localization signal

NO ..... Nitric oxide

$\bullet O_2^-$ ..... Superoxide anions

•OH ..... Hydroxyl radicals

$ONOO^-$ ..... Peroxynitrite

p53-AIP1 ..... p53 apoptosis-inducing protein 1

PAD.....Protein arginine deiminase

PBS ..... Phosphate buffered saline

PBST .....Phosphate buffered saline + Tween-20

PCR..... Polymerase chain reaction

p.o.....per os (oral)

pre-miRNA .....precursor miRNA

pri-miRNA ..... primary miRNA

PTM ..... Post-translational modification

PUMA .....p53 upregulated modulator of apoptosis

qPCR.....Quantitative polymerase chain reaction

RA .....Rheumatoid arthritis

Rb..... Retinoblastoma

RISC.....RNA-induced silencing complex

RNA .....Ribonucleic acid

RONS..... Reactive oxygen and nitrogen species

ROS..... Reactive oxygen species

SAR.....Structure-activity relationship

SDS .....Sodium dodecyl sulfate

SEM .....Standard error of the mean

siRNA ..... Short interfering ribonucleic acid

SLE ..... Systemic lupus erythematosus

SOD1..... Superoxide dismutase 1

S phase ..... Synthesis phase

SSBs.....Single-strand breaks

TNFα.....Tumor necrosis factor alpha

U6 ..... Small nuclear protein RNU6B

UC ..... Ulcerative colitis

UTR ..... untranslated region

UV ..... Ultraviolet

WB ..... Western blot

WT ..... Wild-type

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERAL OVERVIEW

Acute inflammation is the body's natural response to foreign pathogens, damaged cells, and other exogenous stimuli. The purpose of inflammation is to rid the body of these irritants and prevent any damage to cells and tissue. To kill or isolate the source of the inflammatory response, several factors are involved in inflammation, including vasodilation, immune cell infiltration, and the release of pro-inflammatory cytokines (1-3). Vasodilation increases blood flow and subsequent migration of pro-inflammatory immune cells (e.g. macrophages and neutrophils), which also can result in swelling, redness, and tenderness at the site of inflammation (3). Since inflammation triggers an innate immune response, pro-inflammatory immune cells release pro-inflammatory cytokines. Cytokines, like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2), are released to induce DNA damage and ultimately kill invading pathogens, allowing for cell and tissue damage to be repaired (4, 5).

Persistent infections or abnormal immune responses can cause chronic inflammation, which can lead to more serious diseases. Chronic and acute inflammation trigger an immune response; however, as the duration of inflammation increases, insufficient repair and regeneration begins to affect surrounding normal cells and tissue. Chronic inflammatory diseases, like Rheumatoid arthritis (RA) and Ulcerative colitis (UC), are marked by inflammatory cell infiltration, high levels of pro-inflammatory cytokines,

and tissue damage (6-8). Certain chronic inflammatory diseases, including UC, increase the risk for the development of tumors in the affected areas (9, 10). Cancers arising from chronically inflamed tissues differ from sporadic cancers, but share many cancer hallmarks, such as uncontrolled cell growth and decreases in tumor suppressors. In UC-associated colorectal cancer (CRC), tumor suppressors that regulate the cell cycle [i.e. p53 and microRNAs (miRNAs)] are frequently mutated or deleted (11-15). UC and UC-associated CRC are also associated with increased levels of Protein Arginine Deiminases (PADs) (7, 16, 17). PADs are a family of enzymes that catalyze citrullination, the conversion of peptidyl-Arginine to peptidyl-Citrulline (18, 19). PADs are inflammation-dependent enzymes since they are present in neutrophils and macrophages and are often activated in inflammatory environments (20, 21). An introduction to the roles Protein Arginine Deiminases (PADs) play in physiological and pathological conditions, as well as the development and current uses of PAD inhibitors, will be discussed in Chapter 2 (22).

Our studies have used Chlor-amidine (Cl-amidine) to study the effects of PAD inhibition. Upon inhibiting PADs, we have shown decreased levels of inflammation, oxidative stress, and DNA damage associated with UC (7, 8). Likewise, subsequent inhibition of tumorigenesis, alongside increases in tumor suppressors, results from Cl-amidine treatment in our cell and animal models of UC-associated CRC (23, 24). An epigenetic study was carried out to further explore and understand how Cl-amidine and PAD inhibition are functioning to prevent the development of tumors in UC-associated CRC. The overall purpose of this dissertation is to prove the efficacy and uncover the mechanisms of action of Cl-amidine by testing this novel therapeutic in models of UC and UC-associated CRC.

1.1.1 Ulcerative Colitis

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease (IBD) that affects approximately 865,000 patients in North America. Each year there are nearly 70,000 new cases in the United States and Canada alone. Northern Europe also has similar incidence and prevalence rates (25). UC affects patients usually between the ages of 15 and 30, but the disease has a secondary peak between the ages of 50 and 70 years (25). The disease is characterized by inflammation of the mucosa of the large intestine with clinical symptoms of rectal bleeding, pain, and diarrhea (26). It is generally unknown what causes UC, but environmental factors, genetics, and abnormal immune responses are thought to play major roles in the development of the disease (25, 26).

Since UC is associated with autoimmunity and inflammatory dysregulation, there are various treatment strategies available. The first line of treatment for mild to moderate cases of UC is to prescribe anti-inflammatory drugs, such as 5-aminosalicyclic acid (5-ASA) or corticosteroids. To combat the inflammatory response, 5-ASA works as an anti-oxidant, while corticosteroids suppress the hyperactive immune system (25, 26). Unfortunately, 5-ASA is often ineffective or may take four weeks or more to achieve remission (27). Similarly, corticosteroids can have only temporary effects; therefore, more potent (and immunosuppressive) options are available. Biologics that inhibit inflammatory targets, for instance tumor-necrosis factor-α (TNF-α), are available after initial treatments are refractory and unsuccessful at inducing remission of active UC (28). However, biologics, including adalimumab (Humira), are associated with dangerous side-effects (28). For severe cases of UC, partial or complete surgical removal of the colon is necessary; yet, this is used as a last resort when other treatment strategies have failed (29).

Understanding the fundamental processes that initiate and perpetuate the active disease state of UC is important to develop safer and more effective alternatives to the current therapeutics.

1.1.1.1 INFLAMMATION

There are several key features that contribute to the severity and progression of UC. The aberrant immune response at the onset of UC leads to the release of pro-inflammatory cytokines. Nevertheless, (controlled) inflammation is a natural, regulatory process in response to certain stimuli. iNOS and Cox-2, key pro-inflammatory cytokines, are regarded as universal indicators of active UC and their expression levels correlate to the severity of the disease (30-36). iNOS is an enzyme that converts L-Arginine to nitric oxide (NO) and is associated with the inflammatory response (2). NO is an important signaling molecule with various effects (e.g. vasodilation (37, 38), immune response (1)). NO is released by stimulated inflammatory cells (e.g. macrophages, monocytes, neutrophils) as a free radical causing lethal DNA damage in invading pathogens (4, 39). Cox-2, an enzyme that converts arachidonic acid into prostaglandins, also works by regulating inflammation and is normally only expressed in cells involved in an inflammatory response (2, 5). Additionally, inflammation is regularly associated with elevated calcium levels due to activated signal transduction cascades and the release of calcium from apoptotic cells (40-42); subsequently, calcium-dependent molecules and processes see a spike in activity. PADs, which catalyze the citrullination of peptidyl-Arginine to peptidyl-Citrulline, are an example of calcium- and inflammation-dependent enzymes that have physiological functions to promote apoptosis (20, 43).

Under prolonged inflammatory conditions associated with UC, physiological inflammatory processes become dysregulated, resulting in various deleterious events, including oxidative stress and the accumulation of DNA damage caused by NO and other free radicals produced by inflammatory cytokines (4, 39, 44). Additionally, Cox-2 upregulation is found in CRC (45) and PADs/excessive citrullination are implicated in chronic inflammatory diseases where elevated calcium levels remain for extended periods (see Chapter 2 for further discussion concerning PADs and inflammatory diseases). These examples further demonstrate how the maintenance of the inflammatory response is essential to avoid tipping the balances towards pathological inflammation.

1.1.1.2 Oxidative Stress and DNA Damage

In response to the inflammatory environment, leukocytes infiltrate and produce reactive oxygen and nitrogen species (RONS) to attack stimuli (46). RONS that are produced by pro-inflammatory leukocytes include: superoxide anions ($\bullet O_2^-$), hydroxyl radicals ($\bullet OH$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) (47-49). Superoxide dismutase 1 (SOD1), Glutathione peroxidase 1 (GPx1), and catalase are important enzymes that catalyze free radicals into stable molecules (50-52). SOD1 targets and converts superoxide anions to hydrogen peroxide and oxygen molecules. Subsequently, GPx1 and catalase function by reducing hydrogen peroxide to more steady water and oxygen molecules.

The presence of RONS leads to oxidative stress if antioxidant defenses are impaired (53). Typically antioxidant enzymes (e.g. SOD1, GPx1, and catalase) are detrimentally repressed in UC, driving oxidative stress (54-57). Then, upon prolonged exposure to oxidative stress, DNA damage can occur (10, 44, 58). Single-strand breaks (SSBs)

commonly result from oxidative stress, but when multiple SSBs occur close by on complementary strands of DNA, double-strand breaks (DSBs) can occur (59, 60). The buildup of unrepaired DNA breaks can result in improper DNA replication with mutations in critical tumor suppressors, oncogenes, and repair genes, ultimately leading to the initiation of carcinogenesis (61).

1.1.2 Ulcerative Colitis-Associated Colorectal Cancer

There are several inflammatory diseases that are known to increase the patients' risk of developing malignancies at the sites of chronic inflammation, including Barrett's esophagus (esophageal cancer) (62, 63), pancreatitis (pancreatic cancer) (64, 65), and hepatitis (liver cancer) (66, 67). Comparably, due to the chronic inflammatory state of UC, patients suffering from UC are at a high risk of developing colorectal cancer (CRC). Approximately 20% of UC patients will develop CRC as the risk increases yearly (9). In recent years, CRC was predicted to cause 50,000 deaths yearly, making CRC the second leading cause of cancer-related deaths (in both men and women) in the United States (68). Once UC-associated CRC is detected, treatments options include surgery to remove the dysplasia/tumorigenic areas (69, 70). However, since UC patients have the heightened potential of developing CRC, there are several cancer preventative strategies available, including scheduled colonoscopies to monitor the extent of UC and to ensure that CRC has not manifested (71, 72). Similarly, current treatments of UC act as chemopreventatives by inhibiting potentially tumorigenic processes, only if remission is achieved and sustained. The prevalence and mortality of CRC, even with current treatment options, further substantiates the need for a successful and nontoxic UC treatment.

1.1.2.1 Cell Proliferation and the Cell Cycle

The cell cycle is divided into 4 active phases: Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). In G1 and G2, cells are growing in order to accommodate DNA replication (S phase) and cell division (M phase) (73). There are checkpoints at G1/S and G2/M to ensure that the cellular genome is stable and that cells are properly prepped to divide (74). The G1 checkpoint is important to prevent the replication of aberrant DNA sequences in cells with damaged DNA (75). Also, to progress through G1 to S, a series of interactions between cyclins and cyclin-dependent kinases (Cdks) are necessary. Cyclin D and Cyclin E are key cyclins in G1/S that correspond to Cdk4/6 and Cdk2, respectively (76). These interactions must both occur sequentially to fully phosphorylate retinoblastoma (Rb) protein and activate transcription factors that progress the cell through G1 to S (77). Since the cell cycle requires Cyclin D-Cdk4/6 and Cyclin E-Cdk2 to advance, cyclin and Cdk inhibitors can induce cell cycle arrest in response to stress (e.g. genomic and oxidative stress) (78, 79). In cancer, these cell cycle monitors are frequently dysregulated, making increased or uncontrolled cell proliferation one of the hallmarks of cancer (80). Therefore, the development of therapeutic cell cycle inhibitors is a noteworthy anti-cancer approach.

1.1.2.2 p53 and Tumor Suppression

p53 is widely accepted as a tumor suppressor or even the "guardian of the genome" (81) and can be activated in response to certain stimuli, such as oxidative stress and DNA damage (82, 83). Upon stimulation by stressors, p53 becomes post-translationally modified (typically by phosphorylation (84) or acetylation (85, 86)), which increases the half-life and accumulation of p53 (87, 88), and becomes localized in the nucleus. Once in

the nucleus, p53 can transcriptionally trigger cell cycle arrest at the G1/S checkpoint to allow for DNA repair. The genes encoding for $p21^{WAF/Cip1}$, a Cdk inhibitor, and growth arrest and DNA damage protein 45 (GADD45) are the main cell cycle targets of p53 that prevent the damaged cells from leaving G1 and entering S phase (89-92). Alongside cell cycle arrest, p53 can activate the DNA mismatch repair gene *MLH1* in response to DNA damage (93). However, in situations where the damaged DNA is irreparable, p53 can initiate apoptosis by inducing its pro-apoptotic targets, including p53 upregulated modulator of apoptosis (PUMA) (94), p53 apoptosis-inducing protein 1 (p53AIP1) (95), and Bcl-2 associated X protein (Bax) (96).

When p53-dependent pathways are no longer needed, a ubiquitin ligase MDM2 binds to and ubiquitinates p53, designating p53 for degradation in the cytoplasm (97). MDM2 regulates p53-induced cell cycle arrest and apoptosis (98, 99), but MDM2 is also considered oncogenic since its overexpression can inhibit p53 function in genotoxic conditions (100, 101). p53 stability and functional maintenance are essential to avoid cancer development resulting from genomic aberrations; yet, p53 activity is diminished during the early stages of UC-associated CRC versus the late stages of sporadic CRC (13, 102, 103). Likewise, p53 variations (i.e. mutations or deletions) are reported in approximately 85% of UC-associated CRC cases (11, 12). With all of the regulatory targets of p53, it is evident the dire consequences that p53 deletion and/or loss of function can have in pathological conditions, like UC and UC-associated CRC.

1.1.2.3 MICRORNA

As a mechanism of tumor suppression, microRNAs (miRNAs or miRs) are short non-coding RNA sequences that work post-transcriptionally to silence target genes. There

are several steps and key machinery involved in the biogenesis of mature miRNAs. Briefly, the initial RNA transcripts of miRNA commonly fold into short hairpin/stem-loops. The sequence of several different mature miRNAs can be found within these primary miRNA (pri-miRNA) structures; nevertheless, the generation of specific mature miRNAs is dependent on the processing. Pri-miRNAs are cleaved in the nucleus by the RNase Drosha to form precursor miRNA (pre-miRNAs) (104). Upon export from the nucleus to the cytoplasm by Exportin-5 (105), the loop of the pre-miRNA is cleaved by Dicer with subsequent strand cleavage (106-108). Mature miRNAs are then incorporated into a RNA-induced silencing complex (RISC) where the miRNA binds to the 3' untranslated region (UTR) of messenger RNAs (mRNAs) that share a complementary sequence (109). MiRNAs that silence tumor-promoting genes are tumor suppressor miRNAs, while miRNAs that silence anti-cancer genes are termed onco-miRs. MiRNA expression, depending on gene targets, can affect cancer initiation, development, and metastasis. The expression profiles of many tumor suppressor miRNAs are downregulated in tumor tissue as compared to normal adjacent or healthy tissue. Examples of downregulated miRNAs in cancer include: miR-16 in leukemia, breast, and colon cancer (15, 110, 111); miR-200b in gastric, breast, liver cancer (112-114); and miR-195 in cervical and bladder cancer (115, 116).

MiR-16 is a putative tumor suppressor miRNA with cell cycle targets, including Cyclin D1/E1 and Cdk6 (23, 117-120). Typical of miRNAs encoded in intronic regions, miR-16 expression is regulated by its host gene *DLEU2* (110, 121). As examples of host gene expression affecting miR-16 expression, chronic lymphocytic leukemia (CLL) patients frequently have *DLEU2* deletions with subsequent miR-16 deletion (110).

Meanwhile, in instances of cancer where *DLEU2* is not genomically lost, miR-16 expression can be downregulated due to aberrations in the *DLEU2*/miR-16 promoter (122, 123). Alternatively, abnormalities in miRNA processing can affect miRNA maturation (124, 125). For instance, p53 is known to interact with Drosha to promote maturation of miRNAs, including miR-16 (124). Since p53 is mutated or deleted in about 85% of cases of UC-associated CRC (11, 12), this provides additional foundation for the prevalence of miR-16 downregulation in UC-associated CRC.

1.1.2.4 DNA METHYLTRANSFERASES (DNMTS)

In addition to expression changes in miRNAs, aberrant DNA methylation is another common epigenetic feature of UC-associated CRC (126). The DNMTs are a family of enzymes that catalyze the addition of methyl groups to cytosines. The enzymatically active members of the DNMT family include the abundant maintenance methyltransferase, DNMT1, and de novo methyltransferases, DNMT3A and DNMT3B (127). DNMT1 only methylates hemi-methylated substrates, while DNMT3A and DNMT3B do not exhibit a preference for hemi-methylated substrates (128). CpG dinucleotides are most commonly the targets of DNA methylation with promoter methylation often resulting in gene silencing (129). This gene silencing could possibly lead to carcinogenesis if the silenced gene is a tumor suppressor (130-132). In the case of miR-200b, hypermethylation of its host gene promoter can even trigger methylation-mediated gene silencing of miRNAs (133). In UC patient samples, there are several genes that are also repressed by promoter hypermethylation, such as the DNA mismatch repair gene *MLH1* (134) and the cell cycle inhibitor p16INK4a (135). With the substantial involvement of DNMTs in the epigenetic

regulation of tumor suppressors, preventing hypermethylation is a potential UC-associated CRC therapeutic avenue.

1.2 *IN VITRO* MODELS

For the *in vitro* models used to study inflammation, oxidative stress, cell proliferation, and epigenetics, we used immortalized human colorectal cancer and mouse macrophage cell lines. HCT116 human colorectal cancer cells were used as a model for colonic epithelial cells, which are the cells that become pro-cancerous in colon carcinomas (136, 137). HCT116 wild-type (HCT116wt) cells are p53 proficient, but there are isogenic lines of HCT116 cells that have been constructed to be p53 deficient (HCT116 p53-/-), DNMT1 deficient (HCT116 DNMT1-/-), and DNMT3B deficient (HCT116 DNMT3B-/-). The HCT116 cells were used in studies to investigate DNA damage resulting from inflammatory and oxidative stress, measure p53-dependent expression of miRNAs and their targets, and explore epigenetic mechanisms of gene regulation.

For inflammatory and oxidative stress studies, ANA-1 murine macrophage cells were used to represent inflammatory cells as would be harvested from animal models of inflammation. ANA-1 cells can be stimulated/activated with interferon-gamma (IFN-γ) to produce an inflammatory response, including the release of cytokines (e.g. iNOS, COX-2, etc.). When HCT116 and activated ANA-1 cells are cocultured, this produces an inflammatory environment for the HCT116 cells, which simulates the environment that colonic epithelial cells are exposed to during periods of active inflammation in UC. HCT116 and activated ANA-1 cells were separated after coculture using CD45+ magnetic microbeads. CD45 is a cell surface marker found on all leukocytes; therefore, ANA-1 cells are CD45+ and HCT116 cells are CD45- (138). The coculture experiments were designed

as a model to study the effects of inflammation and oxidative stress on isolated epithelial cells.

In our experiments using PAD inhibitors (i.e. Cl-amidine), it is important to explain that HCT116 cells exhibit a basal level of citrullination. Meanwhile, different cell lines used by other groups to study the effects of citrullination require induction with calcium ionophores (e.g. HL-60 and HEK 293) or stimulation with lipopolysaccharide (e.g. RAW264.7) to detect citrullinated proteins (139, 140). Since HCT116 cells do not require induction of citrullination, PAD inhibitors can be administered directly to untreated cells, eliminating the risk of off-target results caused by the influx of calcium into the cell associated with calcium ionophore treatment. This makes HCT116 cells an ideal model for studying citrullination, especially in UC and UC-associated CRC.

We used HCT116 (wt, DNMT1-/-, and DNMT3B-/-) cells as our *in vitro* model of epigenetic regulation of miRNA. HCT116 cells are a suitable model for studying the effects of hypermethylation caused by DNMTs because they are susceptible to methylation-mediated gene silencing (141, 142).

In vitro cellular models are suitable models for isolating the effects of pharmacological compounds; however there are several limitations of *in vitro* UC and CRC models. *In vitro* models do not reflect all of stages involved in the progression from inflammation to tumor initiation to metastasis (143), nor do they completely represent all of the complex pathways that are involved and interact in *in vivo* models. Therefore, several *in vivo* animal models have been developed to enable the study of premalignant and malignant stages of CRC. These models, which can be used to study therapeutic and

preventative capabilities of a compound, also are reliable with consistent and reproducible results.

1.3 *IN VIVO* MODELS

Multiple *in vivo* animal models of UC and CRC have been established using genetic, xenograft, and chemically-induced methods with rodents of different background strains. These models can be used to study inflammation (without the presence of tumors), inflammation-associated tumorigenesis, and sporadic tumorigenesis. For our *in vivo* studies, we used the azoxymethane (AOM)/ dextran sodium sulfate (DSS) murine models of UC and UC-associated CRC. AOM is a genotoxic, carcinogenic agent that is given via a single intraperitoneal (i.p.) injection at the initiation of the experiment. DSS is a non-genotoxic agent and is thought to cause colitis by disrupting tight junctions between colon epithelial cells, which in turn increases the permeability of the gut barrier. Although the precise mechanism of action of DSS is unknown, DSS causes irritation to the colonic epithelial cells that stimulates an inflammatory response (144). We used 8-12 week old C57BL/6 male mice in all of our *in vivo* models because this strain has proven susceptibility to DSS-induced colitis (145). To note, all male mice were used in our studies because DSS is shown to have lower efficacy in female mice, perhaps due to estrogen levels interfering with the action of DSS (146). DSS is administered in the drinking water in cycles throughout the experiment. The experimental treatment schedule and duration are dependent on the endpoint to be studied. Hence, experiments aimed at treating UC require a shorter duration than experiments aimed at preventing CRC.

1.3.1 AOM/DSS Mouse Model of Ulcerative Colitis

The DSS murine model of UC is a common model because it shares a comparable etiology, pathogenesis, and clinical outcome to human UC (147). In our animal model of acute UC, we included the single i.p. injection of AOM as an experimental control since our UC-associated CRC model also required the initial AOM injection. As verified by our results, AOM alone did not cause inflammation, nor did it induce tumorigenesis (when given with DSS) in short-term experiments. DSS and Cl-amidine treatments began 7 days after the AOM injection and lasted for 14 days. DSS and Cl-amidine (see Chapter 2 for background regarding Cl-amidine) were both administered to the mice in the drinking water and were refreshed every 2 days to ensure stability of the compounds (Figure 1.1). This short-term model imitates the continuous injury to the colon resulting from the inflammatory state of UC and allows us to test our compound for therapeutic properties.

1.3.2 AOM/DSS Mouse Model of Ulcerative Colitis-Associated Colorectal Cancer

The principle behind the AOM/DSS model of UC-associated CRC is that prolonged exposure to damaging inflammation and oxidative stress leads to DNA damage, which ultimately results in tumorigenesis. The carcinogenic initiation by AOM and promotion by DSS allows for tumors to appear within 10 weeks after the AOM injection, as compared to the 30 weeks it would take for mice given AOM alone to develop tumors (148). In addition to the substantially decreased time for tumor development, the AOM/DSS model of CRC is widely used because it demonstrates a sequence of events that are distinct to inflammation-driven CRC, as compared to sporadic CRC (13). For instance, pro-inflammatory cytokines (e.g. TNF, IFN-γ, NF-κB) increase and p53 levels decrease at early

stages of UC-associated CRC, while inflammatory cytokines are not typically elevated and p53 changes do not occur until the final stage of sporadic CRC (13, 102, 103).

For our AOM/DSS murine model of UC-associated CRC, we began cycles of DSS and Cl-amidine treatments 7 days and 14 days, respectively, after the initial AOM i.p. injection and concluded on Day 70 of the experiment. Cycles of DSS consisted of DSS given for 7 days, followed by 14 days of normal drinking water. These cycles were meant to mimic the inflammatory state of the active disease with periods of remission. At Day 35 (midpoint of the experiment), mice had not developed tumors, but, as expected, inflammation and ulceration was significant in the mice given only DSS. This long-term model of UC-associated CRC allows us to replicate cycles of relapsing, chronic inflammation and measure the events preceding (Day 35) and during (Day 70) tumorigenesis.

1.4 Objective of the Research

Over the past 50 years, investigators have tried various approaches with numerous targets to treat UC. Treatments are designed to either treat the symptoms of the active disease or induce remission by targeting the underlying causes of UC. These approaches include: pro-inflammatory cytokine inhibitors, immunomodulators, and free radical traps. Currently the most common treatment options for UC are: 5-ASA, corticosteroids, and TNF-α monoclonal antibodies/biologics (25, 26). Unfortunately, current treatments for UC do not have optimal results in patients. For instance, 5-ASA is a relatively safe option since it has local effects on the colon, but 5-ASA only has marginal outcomes in UC patients. To increase potency, consequentially, safety is often sacrificed. Such as, Humira,

the FDA-approved TNF-α inhibitor, is immunosuppressive with a substantial list of possible, deleterious side effects (e.g. infection, heart failure, skin cancer) (149-151).

The development of an effective treatment (without the negative side effects) is the aim of many current studies in the IBD field, especially with the link connecting the extended duration of UC to the considerable increased risk of UC-associated CRC. Even if UC-associated CRC does not occur, UC causes a significant decrease in the affected patient's quality of life due to the chronic persistence of the disease. For these reasons, the hunt for novel safe and effective therapeutics towards the cure of UC is crucial. With this as a goal of our research, we evaluated the potential of Cl-amidine as a therapeutic compound to treat UC in Chapter 3. We translated our *in vitro* inflammatory models to an *in vivo* murine model of UC and saw that Cl-amidine inhibited the key steps that cause the progression from an abnormal immune response within the colon to UC-associated CRC (Chapter 3). To build off of our successful results showing that Cl-amidine suppresses UC in our models, we tested Cl-amidine as a chemopreventative compound in our UC-associated CRC models (Chapter 4). Also in Chapter 4, we verified a mechanism of action where the tumor suppressor miR-16 and its cell cycle targets are upregulated upon Cl-amidine treatment *in vivo*. To further understand how Cl-amidine is targeting specific miRNAs, Chapter 5 explores the epigenetic mechanism of Cl-amidine in regards to its ability to prevent methylation-mediated gene silencing of tumor suppressor miRNAs that are typically downregulated in cancer. Since we have determined several mechanisms of action for Cl-amidine and have proven its efficacy against UC and UC-associated CRC, Chapter 6 concludes with a summary of the research project and the future directions to be pursued.

1.5 Specific Aims

A schematic diagram of this project, including specific aims and results, is provided in Figure 1.2. Below, individual aims are described:

Specific Aim 1: To determine the therapeutic efficacy of Cl-amidine in our *in vitro* and *in vivo* models of UC (Chapter 3).

Specific Aim 2: To determine the preventative efficacy of Cl-amidine in our *in vitro* and *in vivo* models of UC-associated CRC (Chapter 4).

Specific Aim 3: To understand the epigenetic mechanisms of Cl-amidine *in vitro* (Chapter 5).

FIGURES

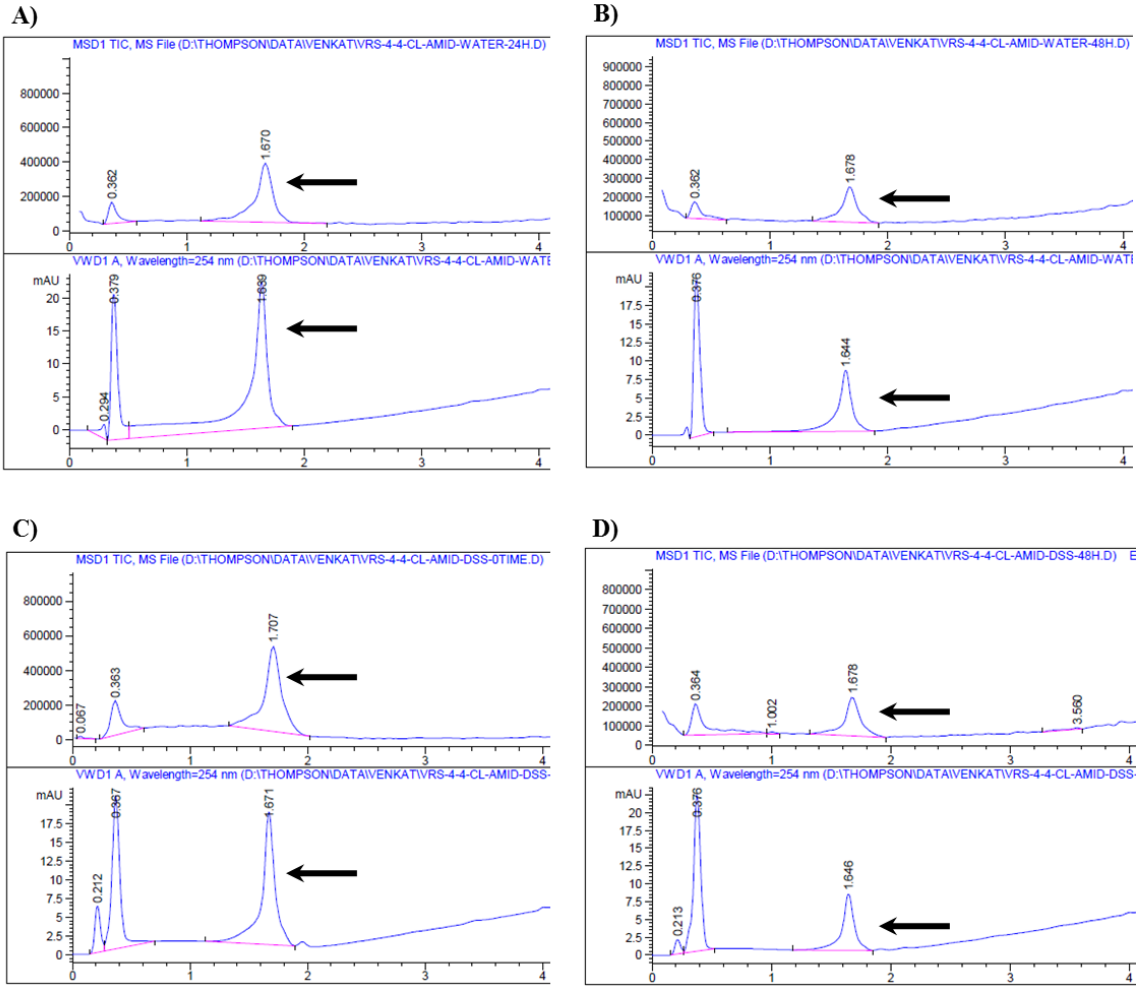

Figure 1.1. Cl-amidine is stable in water and DSS for 48h. As measured by mass spectroscopy by the lab of Dr. Paul Thompson, Cl-amidine remains relatively stable in water for **A)** 24h and **B)** 48h and in DSS + water for **C)** 24h and **D)** 48h. Arrows indicate the peaks for Cl-amidine.

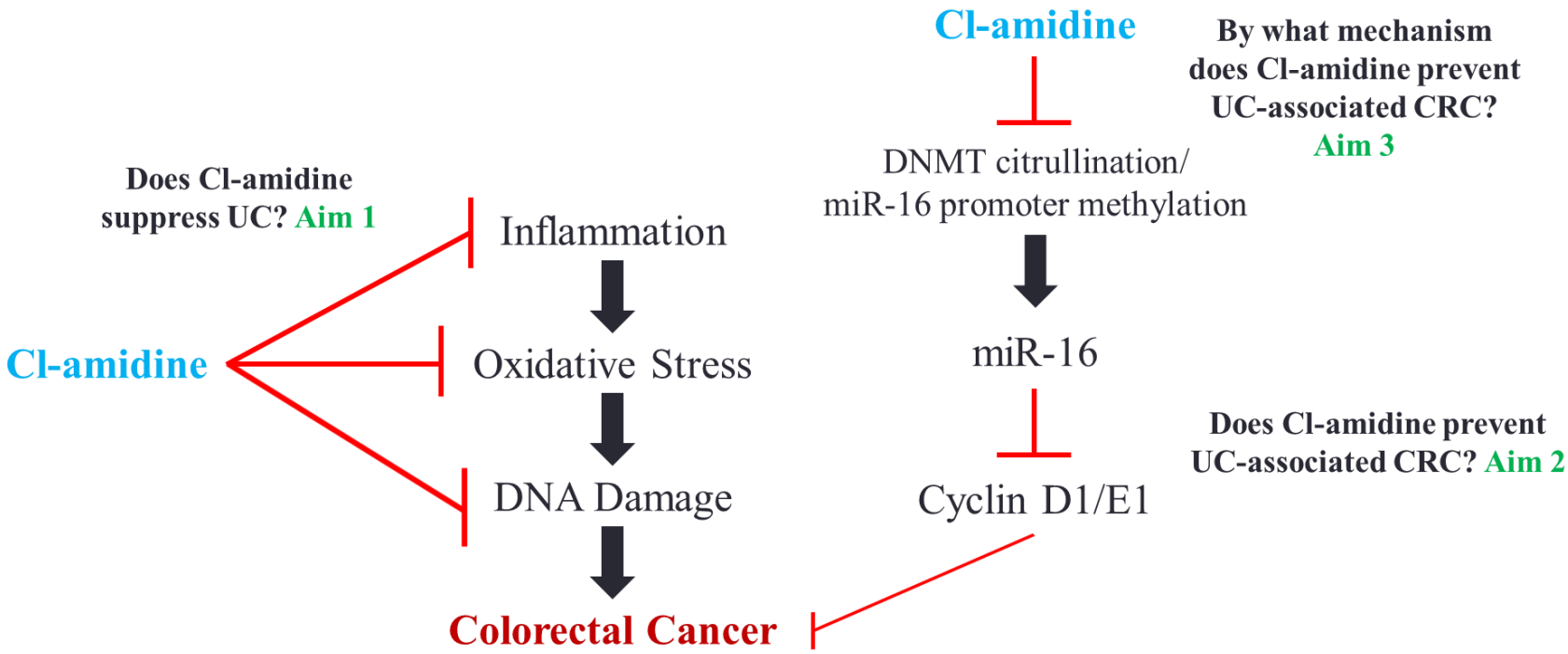

Figure 1.2. Outline of specific aims. Aim 1 will test the efficacy of Cl-amidine to suppress the hallmarks of UC (i.e. inflammation, oxidative stress, and DNA damage). Aim 2 will test if Cl-amidine can prevent UC-associated CRC and will uncover potential mechanisms of action. For a further mechanistic study, Aim 3 will explore how Cl-amidine may epigenetically regulate miRNA promoters by preventing methylation-mediated gene silencing.

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CHAPTER 2

PROTEIN ARGININE DEIMINASES AND ASSOCIATED CITRULLINATION: PHYSIOLOGICAL FUNCTIONS AND DISEASES ASSOCIATED WITH DYSREGULATION[*]

ABSTRACT: Human proteins are subjected to more than 200 known post-translational modifications (PTMs) (e.g., phosphorylation, glycosylation, ubiquitination, S-nitrosylation, methylation, N-acetylation, and citrullination) and these PTMs can alter protein structure and function with consequent effects on the multitude of pathways necessary for maintaining the physiological homeostasis. When dysregulated, however, the enzymes that catalyze these PTMs can impact the genesis of countless diseases. In this review, we will focus on protein citrullination, a PTM catalyzed by the Protein Arginine Deiminase (PAD) family of enzymes. Specifically, we will describe the roles of the PADs in both normal human physiology and disease. The development of PAD inhibitors and their efficacy in a variety of autoimmune disorders and cancer will also be discussed.

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2.1 INTRODUCTION

Posttranslational modifications (PTMs) can have consequential effects on protein structure and function. One such PTM, citrullination, is catalyzed by the Protein Arginine Deiminase (PAD) family of enzymes (1). This enzyme family consists of 5 isozymes (PAD1-4 and 6) (2-4) that have individual tissue specificity and target substrates. PADs rely on increased concentrations of calcium to citrullinate peptidyl-arginine (5). Under physiological conditions in cells, PADs are not normally active until stimulated with calcium. Once stimulated, these enzymes citrullinate a number of structural proteins (e.g., vimentin, filaggrin, and keratin) (6, 7) and proteins involved in the regulation of gene transcription (e.g., histones H1, H2A, H3 and H4) (8-10). Since PADs play functional roles in key cellular processes, their regulation is necessary. There have been several inhibitory compounds developed to study PAD activity. From the parent structures of Cl-amidine and F-amidine, second generation compounds have been synthesized with increased isozyme specificity, *in vivo* stability, and bioactivity (11-13). To date, Cl-amidine is the most widely used pan-PAD inhibitor, while Cl4-amidine and F4-amidine are the most potent PAD3 specific inhibitors (13). Many of these compounds are being tested as therapies and preventatives for numerous diseases where PADs are known to become dysregulated such as diseases of the nervous system, skin, joints, immune system, and colon (14-17). Overall, based on PADs' role in physiological and pathological functions (such as gene regulation and immune response), the field of PAD regulation is gaining traction as a promising target for the treatment and prevention of autoimmune and inflammatory diseases linked to abnormal PAD activity.

2.2 The Protein Arginine Deiminase Family of Enzymes

2.2.1 Citrullination

First described in 1958 by Rogers and Simmonds (1), protein citrullination is the process of converting peptidyl-arginine to peptidyl-citrulline. Since citrulline is a non-genetically encoded amino acid, citrullination occurs post-translationally (18), and because it is a hydrolytic reaction, it results in a 1Da decrease in mass (19, 20). As a result, the strongly basic positively charged side chain of arginine is hydrolyzed by water to form the neutral urea (Figure 2.1). This shift in charge can affect protein-protein interactions, hydrogen bond formation, protein structure, and, in some cases, cause denaturation (13, 21). There is a wide range of cytoplasmic, nucleic, membrane, and mitochondrial proteins that can be citrullinated (22). This review will discuss physiologically targeted protein substrates and citrullination in diseases.

2.2.2 PADs

The PADs were first described in 1977 as the enzymes responsible for this PTM (23) and it is now known that these enzymes are cysteine hydrolases. Citrullination proceeds via nucleophilic attack of a critical active site cysteine on the substrate guanidinium, resulting in the formation of a tetrahedral intermediate that ultimately collapses to form an acyl-enzyme intermediate that is subsequently hydrolyzed to form citrulline (Figure 2.1) (13, 19, 24). While the substrate scope of the PADs remains poorly defined, it is known that these enzymes will not citrullinate free arginines or even methylated arginine in the context of a peptide or protein (18, 19, 25, 26).

2.2.3 PAD Distribution and Structure

There are 5 PAD isozymes found in humans: PAD1, PAD2, PAD3, PAD4, and PAD6 (2-4). Of these isozymes, PAD6 is the only PAD for which no *in vitro* activity has been detected (27). The PAD isozymes have unique tissue localization and overlapping substrates. PADs 1 and 3 are both distributed in the epidermis and hair follicles (28), with PAD1 also localizing in the uterus (2, 29). PADs 2 and 4 have widespread protein distributions. For instance, PAD2 can be located in the central nervous system (CNS), spleen, skeletal muscle, and leukocytes (30-33). PAD4 is found in inflammatory cells (macrophages and neutrophils), mammary gland cells, and tumors (16, 34, 35). PAD6 is localized to eggs, ovary, and the early embryo (4, 35, 36).

Since the crystal structure for PAD4 has been determined, more is known about this isozyme. All mammalian PAD isozymes share 70-95% homology in their amino acid sequence and are approximately 663 amino acids long with a molecular weight of ~74 kDa (Figure 2.2) (18, 24, 37, 38). The PADs have two immunoglobulin-like subdomains at the N-terminus (aa 1-300) and a highly conserved C-terminal domain (aa 301-663) that includes the active site of the enzyme (24). PAD4 also contains a canonical nuclear localization signal (NLS) (aa 56-63) within N-terminal subdomain 1 (Figure 2.2) and is at least partially localized to the nucleus where it deiminates histones H3 and H4 (24, 35). Despite lacking a canonical NLS sequence, PAD2 was also recently shown to localize to the nucleus and citrullinate histone H3 (39).

2.2.4 Calcium Dependency

The PADs are calcium-dependent enzymes (5). There are five calcium binding sites in PAD4, with 2 being located in the C-terminal domain of the enzyme (24) and the

remaining three being present in N-terminal subdomain 2. In the absence of calcium, the active site has a rather open concave structure with a highly acidic region with Cys645, the active site nucleophile, being pointed away from the active site (24, 40). Upon binding calcium, the protein undergoes a series of conformational changes that ultimately results in the movement of key active site residues, including Cys645, into positions that are competent for catalysis (24, 38). Although the pH optimum for PAD activity is 7.6, calcium activation of PAD4 is pH-independent between a pH of 6.0 and 8.5 (25).

2.2.5 PAD Substrate Targets

Not all arginine residues in a protein are equally likely to be citrullinated by PADs. A study investigating the citrullination of filaggrin and trichohyalin, known PAD substrates, was able to determine that approximately 95% and 60% of arginines were converted to citrulline within 3 hours of adding PAD to filaggrin (1:1000 ratio) or trichohyalin (1:30 ratio) substrates, respectively (21). These results demonstrated that the secondary structure of a protein has an effect on the occurrence of citrullination. Filaggrin, which has a secondary structure consisting mainly of a ß-turn, is citrullinated more frequently (~95%) than trichohyalin (60%), which is composed of a single α-helix (19, 21). Likewise, Tarcsa *et al*. (1996) determined the amino acid sequences for filaggrin and trichohyalin and quantified the occurrence of each arginine residue being citrullinated found in each substrate (21). For example, it was found that arginines located next to aspartic acid residues were citrullinated about 80-90% of the time, while arginines found next to glutamic acid residues were rarely citrullinated (0-5%) (19, 21). Furthermore, arginine residues that are located next to an amino group or are flanked by proline are poorly citrullinated (19, 41).

Therefore, depending on the importance, location, structure, and abundance of the arginine residues in a protein, citrullination can have variable effects, such as changes in protein-protein interactions or denaturation (13, 21). PAD1 targets keratin and filaggrin (7); PAD2 targets myelin basic protein (MBP) (30), vimentin (42), actin, and histones (39); PAD 3 targets filaggrin, trichohyalin (7), apoptosis-inducing factor (AIF), and vimentin (43); PAD4 targets multiple proteins involved in gene regulation (histones (24, 44), inhibitor of growth 4 (ING4) (45), p300 (46), p21 (47)) and apoptosis (nucleophosmin (48), nuclear lamin C (49)) ; and there are no known protein substrates of PAD6 (Table 2.1) (35). Several of these protein targets of PAD citrullination will be described throughout the next sections that have a high arginine content (~10%) and/or have particular arginine-rich regions that are critical for their structure and function.

2.3 Normal PAD Function

2.3.1 Normal Cellular Processes Involving PADs

Under physiological conditions, PADs are usually inactive because calcium concentration is maintained at very low levels in the cell (10^{-8} to 10^{-6} M) (19, 50). Normal functioning PADs become activated during certain events, such as apoptosis and terminal epidermal differentiation, where calcium levels are above the physiological concentration (19, 51). Intriguingly, PADs function in gene regulation and other processes at physiological concentrations of calcium; therefore mechanisms to modulate the calcium-dependence of the enzymes must exist (19). In this section, we will discuss four key cellular processes in which protein citrullination is known to play a role.

2.3.2 APOPTOSIS

Calcium acts as a signaling molecule to coordinate apoptosis; therefore, it is needed at high concentrations within apoptotic cells (52). PADs are also activated by these higher concentrations of calcium in cells undergoing apoptosis. However, once activated, it is not fully understood how PADs determine their substrate specificity. One consideration is that PADs may target proteins that are arginine-rich or have arginine-rich regions within their amino acid sequence. In support of this idea, vimentin, an intermediate filament, is found to be citrullinated during apoptosis (51) and Inagaki *et al.* (1989) report that PADs citrullinate the non-α-helical head domain of the vimentin protein (6). The vimentin protein is 466aa long with 9.2% arginine residues (53). In this non-α-helical head domain (aa 2-95), there are 12 arginines that are citrullinated by the PADs (53). When vimentin is citrullinated, the polymer disassembles and the monomers cannot rejoin properly to form the structural support and organelle anchor, triggering structural collapse and further completion of apoptosis (6, 19, 54). For instance, PAD2 can induce apoptosis by citrullinating vimentin, specifically in macrophages. Overexpression of PAD2 leads to vimentin citrullination and apoptosis in Jurkat cells (42).

Likewise, histones and nucleophosmin are known targets of PAD4 and their citrullination can cause the nucleosome and nuclear lamina to collapse, also initiating apoptosis (18, 19). The oligomerization of nucleophosmin is required for localization to the nucleolus and will prevent apoptosis by inhibiting p53 localization to the mitochondria (a key step in apoptosis) (55, 56). Therefore, improper folding/binding caused by citrullination of nucleophosmin can induce apoptosis associated with p53 localization to the mitochondria. Also reported, PAD4 overexpression upregulates p53 and stimulates

mitochondrial-associated apoptosis (57). Additionally, Mizoguchi *et al.* (1998) reported the citrullination of a 70kDa nuclear protein localized on the periphery of the nucleus was linked to dissociation of the nuclear lamina during apoptosis (58). Recently, PAD3 was found to be necessary for apoptosis-inducing factor (AIF)-mediated apoptosis (43). Overall, PAD activation (due to amplified calcium levels during the early stages of apoptosis) can facilitate apoptotic cell death by citrullinating several nuclear and cytoskeletal proteins that can cause structural changes resulting in the disintegration of secondary and tertiary protein structures (6, 18, 19).

2.3.3 Structural Support

Additionally, PADs are involved in the organization of structural proteins in cells undergoing terminal epidermal differentiation (19, 59). This differentiation is mediated by calcium, so there are usually higher concentrations of calcium, which in turn activates the PADs. Within the epidermis, PADs can citrullinate structural proteins, such as, keratin, filaggrin, and vimentin. Filaggrin, as an example, is 4061aa long with a relatively high arginine content (10.8%) (53). Upon calcium-mediated terminal differentiation of epidermal cells, activated PADs can citrullinate these proteins, causing partial unfolding, which makes these proteins more susceptible to protease degradation (19, 60).

2.3.4 Gene Regulation

In cells not undergoing apoptosis, PADs can regulate gene expression (10, 35, 44-47, 61-63). Most research on PAD involvement in gene regulation has been performed on PAD4. One of the best studied systems is the PAD4-mediated regulation of the p53 pathway. For instance, PAD4 citrullinates ING4 protein (45) and once modified, this protein does not bind to p53 and is more prone to protease degradation. Without the

interaction of ING4, the transcriptional targets (e.g. p21) of p53 are suppressed (Figure 2.3A) (45). Another mechanism involves the citrullination of methylated arginine on histones. In this mechanism, PAD4 is recruited to gene promoters where it mediates the citrullination of histone H4 at R3 and histone H3 at R2, R8, and R17 and the modification is associated with gene repression (44). As an example, PAD4, which interacts with the regulatory domain of p53, targets p21 at the p53-binding sites on the promoter region (47). This interaction causes citrullination of histones on the p21 promoter region, ultimately repressing p21 transcription since p53 cannot properly bind to the p21 promoter region (Figure 2.3B) (47). This impaired binding is due to the changes in charge that can affect protein-protein interactions and hydrogen bond formation. Also, citrullination of the histones in the p53-binding sites on the promoter region may cause conformational changes that sterically hinder p53 binding. Li *et al.* (2010) correspondingly reported that PAD4 citrullinated histones on p53 target gene promoters, but they further found that PAD4 relied on the histone deacetylase, HDAC2, to repress the gene expression of p53 targets (Figure 2.3B) (61). It is still unknown how PAD4 is recruited to specific promoters or even how they are activated at physiological calcium levels (35, 62, 63). Wang & Wang (2013) propose a mechanism where PAD4 binds to the promoter region of p53 and represses target gene expression by histone citrullination during periods where the damage response pathways are not needed. Then, when these pathways are needed (i.e. in response to DNA damage), PAD4 dissociates from the promoter and the target genes are activated once again (35). There is also evidence that PAD4 can act as a transcriptional coactivator. In this process, PAD4 citrullinates the Arg2142 in the GRIP1 binding domain of the transcriptional coactivator p300. Once p300 is citrullinated, the interaction between it and

GRIP1, another coactivator, is enhanced (Figure 2.3C) (46). Perhaps, in this case, the neutral charge of citrulline could be more complementary for the interaction between GRIP1 and p300. How the PADs get activated to modulate gene transcription is not known, but György *et al.* (2006) hypothesized that PADs may be active with strict substrate specificity at low levels of calcium and this could explain PAD involvement in gene regulation (19). However, this is unlikely to be correct because different substrates have similar calcium dependencies (62).

PAD4 also plays a role in pluripotency as a transcriptional regulator of several key genes. PAD4 upregulates pluripotent markers (e.g. Klf2, Tcl1, Tcfap2c, Kit, Nanog) and downregulates differentiation markers (e.g. Prickle1, Epha1, Wnt8a) (8, 64). In fact, citrullination of histones H1 and H3, by PAD4, regulates the induction of pluripotency and reprograming of stem cells during early embryonic development (8, 64). Slade *et al.* (2014) also suggest that other PAD isozymes (PAD1-3) may impact pluripotency at other stages of differentiation since they showed individual expression in embryonic stem cells and induced pluripotent cells (8, 64).

2.3.5 Immune Response

PADs 2 and 4 are involved in inflammatory immune responses. PAD4 is mainly expressed in macrophages, neutrophils, and eosinophils whereas PAD2 is highly expressed in macrophages (16). In macrophages, PAD2 becomes activated due to increased levels of calcium (65). Also, PAD4 is involved in the citrullination of histones in neutrophils. After activated PAD4 has translocated to the nucleus of neutrophils, hypercitrullination of histones can trigger the production of neutrophil extracellular traps (NETs) that trap bacteria and other pathogens. The release of NETs can provoke an autoimmune response

to NET-associated nuclear antigens and granule proteins, leading to a pro-inflammatory form of programed cell death termed 'NET-osis' (40, 66, 67).

2.4 PAD INHIBITORS

2.4.1 DEVELOPMENT OF F-AMIDINE, THE FIRST BIOACTIVE PAD INHIBITOR

Thompson and colleagues reported the first bioactive PAD inhibitor in 2006 when they described the synthesis and characterization of F-amidine. The structure of F-amidine is based on benzoyl-arginine amide (BAA), one of the best small molecule substrates identified for the PADs (25, 68). Since the PADs preferentially hydrolyze a positively charged guanidinium, Thompson hypothesized that replacement of one of the amino groups with a methylene fluoride would generate a compound that could covalently modify the enzyme (Figure 2.4A) (68). The resulting fluoroacetamidine containing compound maintains the positive charge and most of the hydrogen bonding capabilities of the substrate guanidinium, but due to the electron withdrawing nature of the fluorine can undergo reaction with a cysteine residue present in the active site of the enzyme (68). This compound, F-amidine, inhibits PAD4 with a k_{inact}/K_I value of 3000 $M^{-1}s^{-1}$ and was the most potent pan-PAD inhibitor at the time. Further experiments demonstrated that F-amidine irreversibly inhibits PAD4 by modifying Cys645, the active site cysteine (69). F-amidine is bioavailable and has been used to confirm that PAD activity can inhibit gene expression (68).

2.4.2 CL-AMIDINE

Building on the success of F-amidine, Thompson and colleagues next synthesized a series of compounds in which they optimized the identity of the warhead by replacing the fluorine with either a chlorine or hydrogen. They also optimized the side chain length

by synthesizing compounds in which the side chain is varied between two and four methylene units (69). From these compounds, Cl-amidine was found to be the most potent compound with a k_{inact}/K_I value of 13000 $M^{-1}s^{-1}$ for PAD4 (69). Subsequent work showed that it inhibits all four active PAD isozymes with similar potencies (11). Cl-amidine is structurally identical to F-amidine except that it has chlorine substituted for the fluorine in the haloacetamidine warhead (Figure 2.4B) (11, 69). By contrast, compounds that lacked an electron withdrawing group or had shorter or longer side chains were rather poor PAD inhibitors (69).

Cl-amidine, like F-amidine, preferentially inhibits the activated, calcium-bound form of the PADs (69) and based on detailed mechanistic studies (13) both compounds irreversibly inhibit the enzyme via nucleophilic attack on the amidine carbon, which results in the formation of a tetrahedral intermediate that is stabilized by proton donation from His471, the general base in the normal PAD-catalyzed reaction. The sulfur then attacks the halide bearing carbon to form a three member sulfonium ring. Proton abstraction and collapse of the tetrahedral intermediate cleaves the sulfonium ring to form the stable thioether adduct (40).

Since its development, Cl-amidine has been the most widely used PAD inhibitor and studies indicate that it is bioavailable and can inhibit PAD4 activity found in the nucleus (69). Remarkably, Cl-amidine was cytotoxic to cancer cell lines (MCF-7, HL-60, and HT29) and showed minimal cytotoxic effects on noncancerous cell lines (NIH 3T3 and HL-60 granulocytes) (70). Furthermore, Cl-amidine triggered the differentiation of the leukemic HL-60 cell line into HL-60 granulocytes (70). As will be discussed later on in

this review, Cl-amidine has been tested on numerous disease models and shows promise for the treatment and/or prevention of many diseases.

2.4.3 Other PAD4 Inhibitors

In order to detect other PAD4 inhibitors, a competitive screening assay was developed using Rhodamine-conjugated F-amidine. This assay overcomes the challenges of traditional PAD activity assays because it does not require strong acids, high temperatures, and toxic reagents that are normally necessary to detect the citrulline product of the reaction (71). Using this assay, multiple drugs that are typically used to treat RA were evaluated for their ability to inhibit PAD4. Streptomycin was found to be a competitive inhibitor whereas minocycline and chlorotetracycline were found to be mixed inhibitors that did not bind to the active site (71). These drugs can potentially provide scaffolds for the next generation of PAD inhibitors (71, 72).

2.4.4 PAD Specific Inhibitors

In disease, it has been shown that only certain PADs may be dysregulated (e.g. PAD4 in RA); therefore, the development of PAD specific inhibitors has been explored. Structure activity relationships (SAR) were generated for F-amidine and Cl-amidine to identify modifications that would increase interisozyme selectivity. One outcome of these efforts was the identification of o-F-amidine and o-Cl-amidine, which contain an ortho-carboxylate on the benzoyl ring of the parent structures (11). Both compounds are significantly more potent than the parent compounds and also exhibited PAD specificity. For example, o-F-amidine selectively inhibits PAD1, whereas o-Cl-amidine preferentially inhibits PADs 1 and 4 (11). Additional studies identified D-o-F-amidine and D-Cl-amidine as a pair of PAD1 specific compounds that possess increased *in vivo* stability (12). As of

2010, Cl4-amidine and F4-amidine, which were tested previously on PAD4, are the strongest PAD3 specific inhibitors (13). TDFA, a tripeptide composed of threonine, aspartate and F-amidine is the most potent PAD4 specific inhibitor to be reported (73). Additionally, in 2005, Kearney *et al.* showed that different metals inhibit PAD activity. In their studies, they found that manganese, samarium, and zinc did not allow the PADs to structurally change into the catalytically active form in the presence of calcium (25). These findings are another step in the development of new and more specific PAD inhibitors.

2.5 PAD Dysregulation in Diseases

2.5.1 Diseases Associated with PAD Dysregulation

PADs have a role in maintaining many vital cellular processes; therefore, it is necessary for a balance in PAD activity or these processes can become uncontrollable with potentially deleterious side effects. Due to this delicate balance, it is not hard to believe that there are numerous diseases associated with PAD dysregulation and abnormal levels of citrullination. In this section, we will discuss our current understanding of the role that PADs play in these diseases even though much (i.e. specific cause for dysregulation) is still to be discerned. We will also discuss specific PAD isozymes that are associated with certain types of diseases, such as PAD1 is linked to psoriasis (15) and PADs 2 and 4 are heavily involved with neurodegenerative and inflammatory (16, 17) diseases and cancers (34).

2.5.2 Causes of PAD Dysregulation

Not much is known about what causes PADs to become dysregulated, but there are several proposed scenarios that may lead to abnormal levels of citrullinated proteins. For example, (i) PAD activity may become uncontrolled at extreme levels of calcium and may

lose target specificity (19). The lack of target specificity could cause loss of activity and even total denaturation in arginine-rich proteins. As well, (ii) increased translation of PADs could also explain for increased levels of citrullination; however, it is unknown what might cause the increased translation (19, 74). (iii) There is abnormal TNFα signaling in Rheumatoid Arthritis (RA) and Ulcerative Colitis (UC), TNFα can induce PAD4 translocation (75), and PADs can citrullinate TNFα (76). Taken altogether, perhaps RA and UC result, in part, from PAD dysregulation (17). Finally, (iv) PAD4 has been seen to autocitrullinate and this may be a form of self-regulation. Although autocitrullination is not shown to interfere with specificity or activity levels of the enzyme, it does affect the enzyme's interaction with other proteins involved in the PTM of histones (62). Once again, as discussed in Section 2.5, the amino acid sequence and structure surrounding arginine residues can also influence susceptibility to citrullination (19, 21).

2.5.3 PADs IN THE CNS

2.5.3.1 NORMAL FUNCTION

PAD2 is the predominant isozyme found in the nervous system (14, 30, 35). While PAD4 does not normally target CNS substrates, it has been discovered in the cytoplasmic granules of neuronal cells (77). PAD2 and 4 are normally in the inactive state in the adult CNS (14), but are active during development (78).

2.5.3.2 ALZHEIMER'S DISEASE

PAD 2 and 4 become activated in the CNS during neurodegenerative processes (14). Alzheimer's disease is linked to an increase in abnormal accumulation of misfolded proteins in the hippocampal region of the brain. Interestingly, an increased amount of PAD2 and citrullinated proteins are found in the hippocampus of patients suffering from

Alzheimer's (14). The structural proteins vimentin and glial fibrillary acidic protein (GFAP) are some of the proteins found to be citrullinated in the hippocampus (19, 38, 79, 80). Likewise, PAD4 and citrullinated proteins were colocalized in regions of neurodegeneration and inflammation in Alzheimer's patients (77).

2.5.3.3 Prion Disease

Similar to Alzheimer's disease, prion diseases involve misfolded proteins alongside deterioration of many structures in the brain (79). Calcium levels are dysregulated in these diseases and are likely the reason for high amounts of active PADs and citrullinated proteins in the CNS (79).

2.5.3.4 Multiple Sclerosis

In the CNS, the MBP is essential for maintaining the myelin sheath, which is involved in neuronal signal transduction throughout the CNS (19, 79). Signal transduction requires the myelin sheath to act as an insulator with the MBP holding a positive charge (18). In Multiple Sclerosis (MS), the myelin sheath is degraded, leading to decreased signaling. PAD isozymes 2 and 4 are involved in the pathology of MS (19, 30, 35, 38, 75). It is thought that PAD4 is brought to the CNS by infiltrating macrophages during diseased states and may citrullinate proteins that are not naturally targeted in the CNS (81). The excessive citrullination of the MBP is thought to be a major contributor to the disease (19, 30, 38, 82). It has been established that up to 90% of MBPs are citrullinated in severe forms of the disease (38, 83). Citrullination causes MBP to partially unfold and make them more susceptible to degradation by proteases, like cathepsin D (19, 83, 84). Also the change from positively charged arginine to neutral citrulline is not optimal for the electrical specifications required for proper signal transduction (19, 84). The high frequency of

arginine residues (10.3%) in the amino acid sequence of MBP may also explain why it is targeted by PADs (85). While the PAD2 knockout still develops experimental autoimmune encephalomyelitis (EAE), a preclinical animal model of MS, overexpression of PAD2 gives MS-like symptoms, suggesting that multiple PAD isozymes are involved in the etiology of this disease (86).

2.5.4 PADs IN THE EPIDERMIS

2.5.4.1 NORMAL FUNCTION

PAD1 is highly expressed in the epidermis where it citrullinates keratin K1and filaggrin (7, 19, 35, 38). These proteins aid in the maintenance of homeostasis and structural flexibility in the dermis and epidermis (7, 18, 38). In normal skin, keratin K1 is citrullinated at low levels and degraded to prevent cornification.

2.5.4.2 PSORIASIS

Psoriasis is a type of dermatitis characterized by excessive proliferation and atypical epidermal differentiation which contribute to the flaky, dry patches of skin (19, 38). The possible cause of the disease can be explained due to the fact that there is no citrullinated keratin K1 found in skin samples from patients with psoriasis (15, 18, 19, 38). The lack of citrullinated proteins causes excessive cornification and an inflammatory response (15, 18, 19, 35, 38). Psoriasis is the only disease linked to PAD1 dysregulation and it is unknown what triggers its decreased enzymatic activity (15, 18, 35, 38).

2.5.5 PADs IN THE SYNOVIAL JOINTS

2.5.5.1 NORMAL FUNCTION

PAD2 and PAD4 mRNA and protein are expressed in peripheral blood from both RA patients and healthy control patients (16). In healthy patients as compared to the RA

patients, the PAD isozymes were inactive since the monocytes and macrophages, where the PADs were located, were not involved in an inflammatory response (16). Also, there were fewer macrophages and monocytes found in the joints in healthy patients. These PAD enzymes are typically dormant in the leukocytes and synovial joints until they are activated during an inflammatory immune response.

2.5.5.2 RHEUMATOID ARTHRITIS

RA is a chronic inflammatory disease of the joints that is thought to be caused or intensified by PAD dysregulation. In patients with RA, an increase in PAD2 and 4 translation is observed alongside increased enzyme presence and activation (19, 74). Vossenaar *et al.* (2004) developed a model for PAD infiltration into the synovial joints of RA patients where PAD2 and PAD4 are expressed in monocytes and macrophages that are recruited to the joint (16). Their proposed model shows that the activated macrophages and monocytes, containing the inactive PADs, are eventually degraded in the joints and undergo apoptosis where the PAD isozymes become activated as calcium levels rise. PAD activation then causes cellular protein (e.g. vimentin) citrullination and the PADs can seep out of the cell to target extracellular proteins (16). For example, PAD2 and 4 can citrullinate fibrin in the affected joints, producing an immune response. Also, PAD4 has been shown to citrullinate antithrombin, a thrombin inhibitor. Thrombin is involved with the onset of RA; therefore, the citrullination of antithrombin may further lead to RA (87).

In many inflammatory diseases, such as RA, inflammation is a result of an atypical immune response to bacteria and other stresses (e.g. citrullination) (19, 88). NETs are found to be dysregulated in RA, causing an increase in antibodies against citrullinated proteins (89). Correspondingly, there are high levels of antibodies against citrullinated

proteins found in the blood of RA patients, possibly explaining the atypical immune and inflammatory responses correlated with RA (18, 89). Importantly, the presence of these antibodies is the most specific diagnostic for the disease and they are present in the sera of RA patients 4-5 years, on average, before clinical symptoms appear (90). This suggests that dysregulated PAD activity is potentially disease initiating. Despite the specific causes of RA still being unknown, there have been several haplotypes of PAD4 that are correlated with an increased risk of developing the disease (91).

2.5.6 PADs and Their Role in NET Formation

As mentioned in Section 3.5, PAD4 is involved in a unique form of cell death, 'NETosis'. Released NETs actively trap bacteria and other pathogens that prompt this type of immune response. This immune response is a common defense mechanism against foreign microbes. The effects of NET dysregulation play a role in a number of other autoimmune diseases, including psoriasis (92), systemic lupus erythematosus (SLE) (93), and RA as previously discussed. Interestingly, pro-inflammatory IL-17 is released during NET formation in psoriasis, SLE, and RA, indicating that this aberrant immune response triggers an inflammatory response (89, 92, 94).

2.5.7 PADs in the Colon

2.5.7.1 Normal Function

There is no data on the normal function of PADs in the human colon. Perhaps the presence and dormancy of PADs in normal colonic tissue resembles that in the healthy, non-arthritic patients, as described in Vossenaar *et al.* (2004), where the inactive PADs are present in macrophages and monocytes (16).

2.5.7.2 Ulcerative Colitis

UC is another inflammatory disease where PADs 2 and 4 are involved in the pathogenesis of the disease. UC is characterized by chronic, relapsing periods of inflammation in the colon. The exact cause of the disease is unknown but it has been proposed that an atypical immune response is triggered by an inflammatory response to several cytokines produced by activated macrophages (88). In UC, similar to RA, high levels of anti-citrullinated protein antibodies are found in the blood (17). Thus, UC could be caused by an autoimmune response to improperly folded proteins caused by excessive citrullination. The specific PAD4 haplotypes that have been linked to genetic susceptibility of RA have similarly been linked to UC (91).

2.5.8 PADs in Cancer

PAD dysregulation is also associated with cancer (34). For example, PAD2 levels are elevated in luminal breast cancer cell lines (95). Likewise, since PAD4 regulates gene expression as a corepressor, its dysregulation is thought to be linked to various cancers, such as breast, renal, and colorectal cancer (34, 47). Chang *et al.* (2009) found that PAD4 protein and mRNA expression levels were significantly increased in more than 12 types of cancers, including breast, lung, liver, esophageal, colorectal, renal, bladder, and ovarian malignant tumors collected from patients (34). There were elevated levels of PAD4 found in these cancerous tissues, similar to the increased PAD4 levels in the tissues of patients suffering from inflammatory diseases (i.e. RA, UC) (34, 96). Interestingly, in benign tumors and non-cancerous inflamed tissue samples, PAD4 was not expressed, except tissue samples from the gastrointestinal tract (34). These higher PAD levels in tumor versus non-tumor tissues suggest that abnormal PAD activity/citrullination can cause a dysregulation

of gene expression. When tumor suppressor genes are the targets of PAD4 dysregulation, tumorigenesis can occur. In contrast to results indicating that PAD4 overexpression upregulates p53-mediated apoptosis, PAD4 has been shown to promote tumorigenesis by suppressing p53 and disrupting the cell cycle and apoptosis (57). Similarly, PAD4 citrullination of the intermediate filament, cytokeratin, is determined to interrupt apoptosis (57). Overall, these findings support the premise that PADs promote tumorigenesis.

Alternatively, Stadler *et al.* (2013) reported that PAD4 negatively regulates tumor invasiveness in breast cancer models *in vitro* and *in vivo* (97). In this study, PAD4 citrullinated glycogen synthase kinase-3ß (GSK3ß) in MCF-7 breast cancer cells and in a tumor xenograft animal model. Upon PAD4 downregulation, there was an increase in epithelial-to-mesenchymal transition (EMT) and tumor invasiveness (97). These results indicate a complex role of PAD4 in tumorigenesis with contradicting roles between various cancer types.

2.5.9 PAD Inhibitors Used in Diseases

In addition to using PAD inhibitors to study PAD biology, many labs that work on models of diseases caused by PAD dysregulation are testing the potency and safety of these inhibitors to treat and prevent their disease of interest. For example, 2-chloroacetamidine was found to reverse the effects of PAD activity in numerous mouse models of MS (98). Likewise, current inhibitors that show efficacy against PAD2 and especially PAD4 have been tested on models of RA. Excitingly, Cl-amidine, a pan-PAD inhibitor, was found to reduce clinical symptoms and disease severity in an *in vivo* mouse model of RA (99).

The progress made in using PAD inhibitors for the treatment of RA should be translatable to other hyperactive immune and inflammatory diseases associated with PAD

dysregulation. As evidence of translation to other diseases, Cl-amidine was found to prevent and treat UC when given in a mouse model of UC (17). Additionally, Cui *et al.* (2013) reported that Cl-amidine induced the upregulation of several tumor suppressor microRNAs, in HCT116 colon cancer cell line, that are reportedly downregulated in cancers (e.g. miR-16, let-7) (100-103). Cl-amidine also shows proven efficacy in a multitude of other disease models including: cancer (70, 100, 104), SLE (105), a spinal cord injury model (106), atherosclerosis (107), and hypoxia (108). Altogether, these findings demonstrate the potential for PAD inhibitors as disease treatments and preventatives.

2.6 CONCLUSION

As discussed in this review, the Protein Arginine Deiminases catalyze the citrullination of protein embedded arginine residues, a PTM that has many functional implications on target proteins. Citrullination by PADs affects vital cellular responses involving cell death (via apoptosis and NET-osis), terminal epidermal cell differentiation, and gene regulation. PADs have complex roles in these processes and the particular regulatory mechanisms that control PAD activation/inactivation need to be explored. To better understand the mechanisms and physiological functions of PADs, a series of potent pan-PAD and isozyme-specific inhibitors have been used. Likewise, these PAD inhibitors have been tested on *in vitro* and *in vivo* disease models that are associated with aberrant PAD regulation. Many of these studies have seen success in treatment and/or prevention of diseases including: rheumatoid arthritis, multiple sclerosis, ulcerative colitis, and cancer. Future research on typical PAD activity and atypical involvement in disease will certainly

continue to expand as more encouraging results spotlighting effective PAD regulation are published.

ACKNOWLEDGEMENTS

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TABLES

Table 2.1. PAD target substrates. Known substrates that are targeted by the individual isozymes of the PAD family of isozymes.

Isozyme	Substrates
PAD1	Keratin, filaggrin [7]
PAD2	Myelin basic protein [30], vimentin [42], actin, histones [39]
PAD3	Filaggrin, trichohyalin [7], apoptosis-inducing factor, vimentin [43]
PAD4	Histones [24, 44], ING4 [45], p300 [46], p21 [47], nucleophosmin [48], nuclear lamin C [49]
PAD6	None known

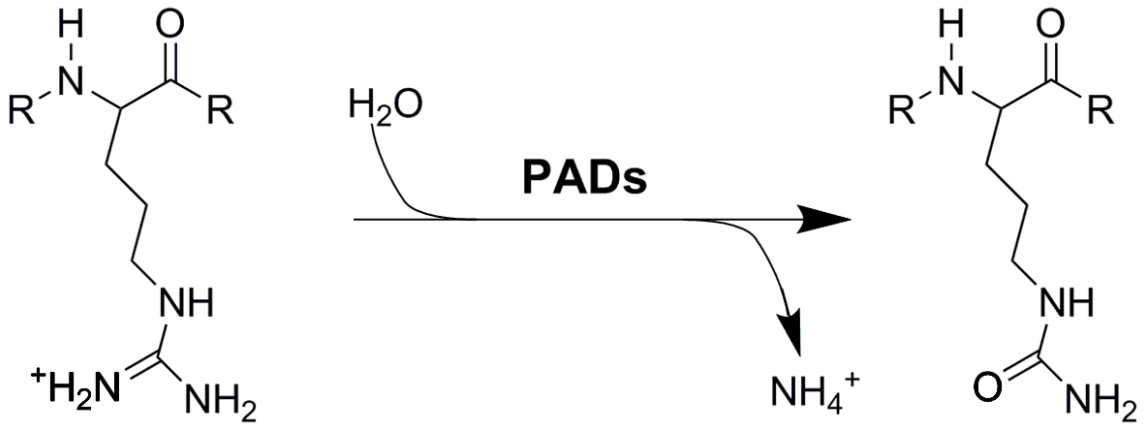

Figure 2.1. The process of citrullination. The primary amine group of peptidyl-arginine is hydrolyzed upon interaction with the cysteine of the deiminating enzyme and is replaced to form a keto group. The end products of this reaction are peptidyl-citrulline and free ammonium.

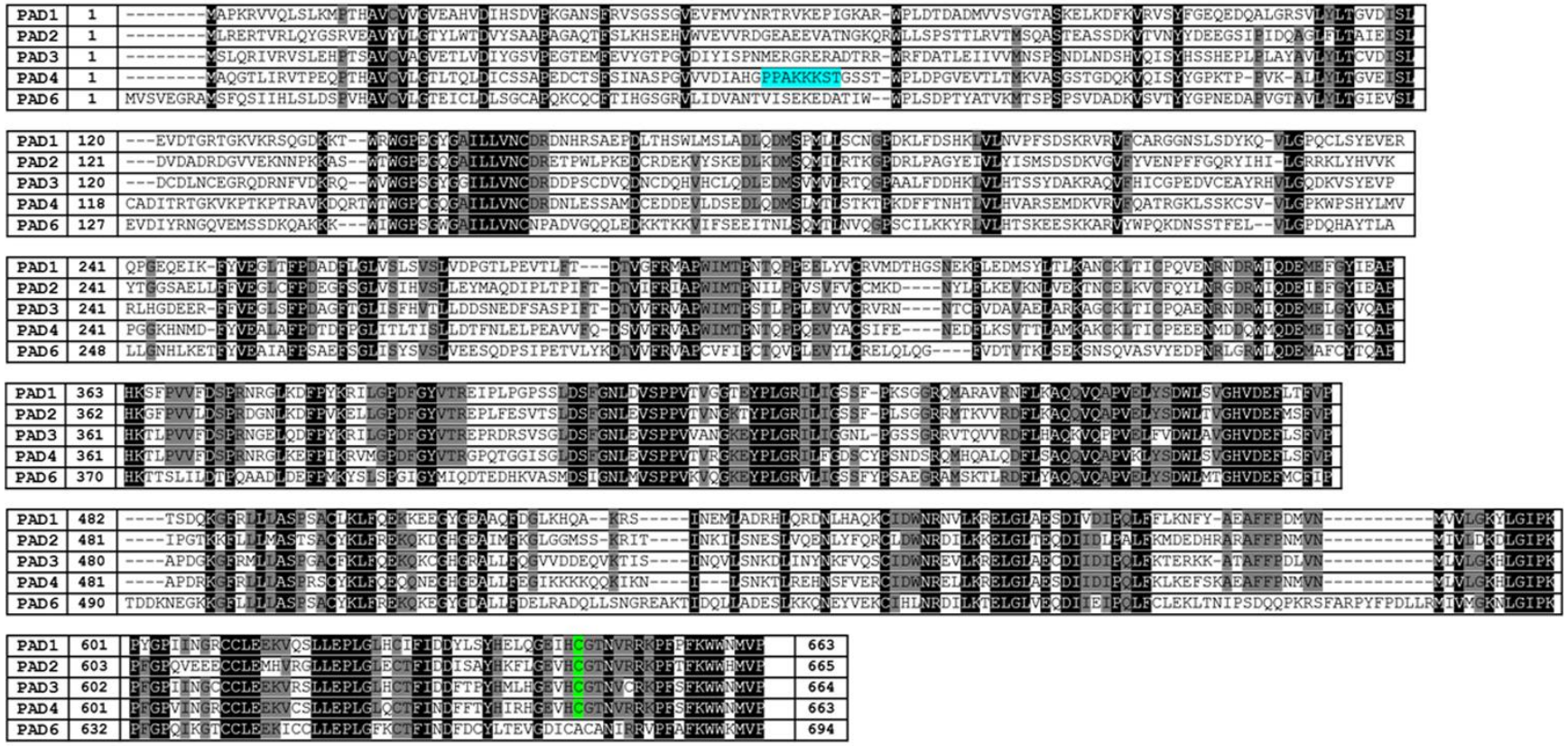

Figure 2.2. Human PAD isozyme sequence alignments. Amino acid sequence alignment of the PAD isozymes with approximately aa 1-300 and 301-663 containing the N-terminus and C-terminus domains, respectively. Highlighted in black are amino acids that are conserved in all 5 isozymes, while gray highlighted amino acids are conserved in 4 of the 5 isozymes. In PADs 1-4, the important cysteine residue (Cys645) in the active site is highlighted in green and, in PAD4, the nuclear localization signal (NLS) sequence is highlighted in blue.

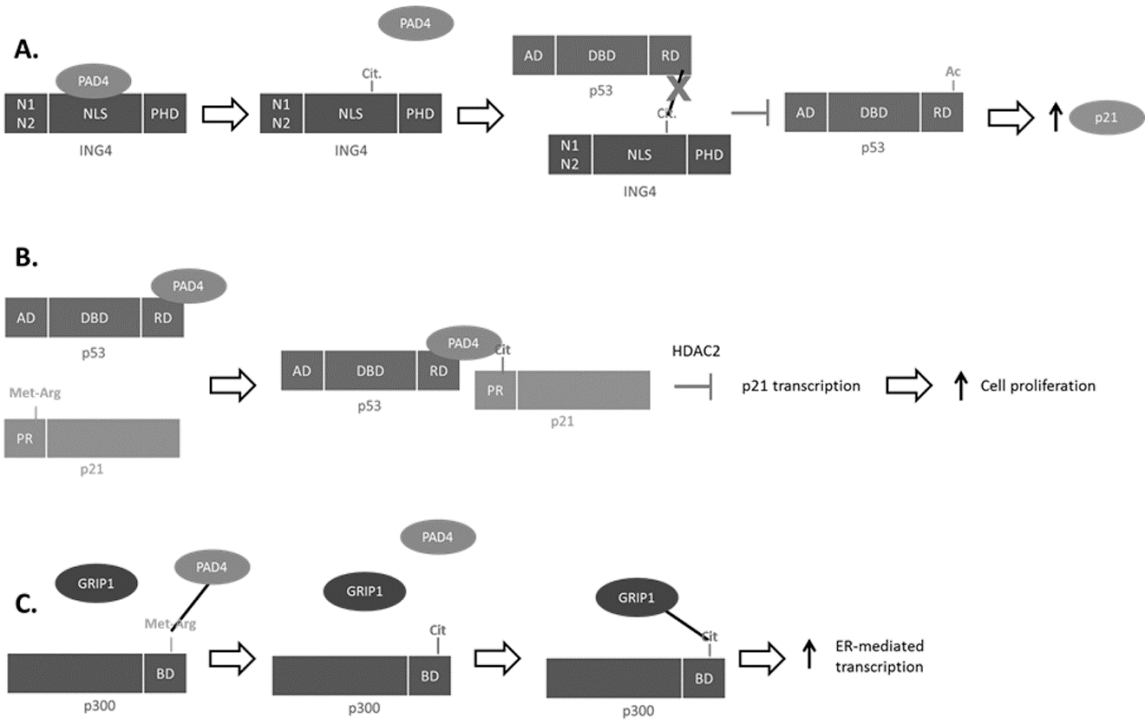

Figure 2.3. Mechanisms of gene regulation involving PADs. A) Activated PAD4 citrullinates ING4 at the nuclear localization signal (NLS) region. Therefore, the regulatory domain (RD) of p53 cannot bind to the NLS of ING4, leading to inhibition of p53 acetylation and inhibition of subsequent upregulation of p21. **B)** PAD4 interacts with the regulatory domain of p53. The PAD4 attached to p53 is recruited to the promoter region (PR) of p21 where PAD4 citrullinates methylated arginine on histones at the promoter region. This citrullination, along with HDAC2, inhibits p21 transcription, resulting in an increase in cell proliferation and inhibition of cell cycle arrest. **C)** PAD4 citrullinates methylated arginine on the GRIP1 binding domain (BD) of p300. This leads to increased binding to GRIP1 and results in increased ER-mediated transcription.

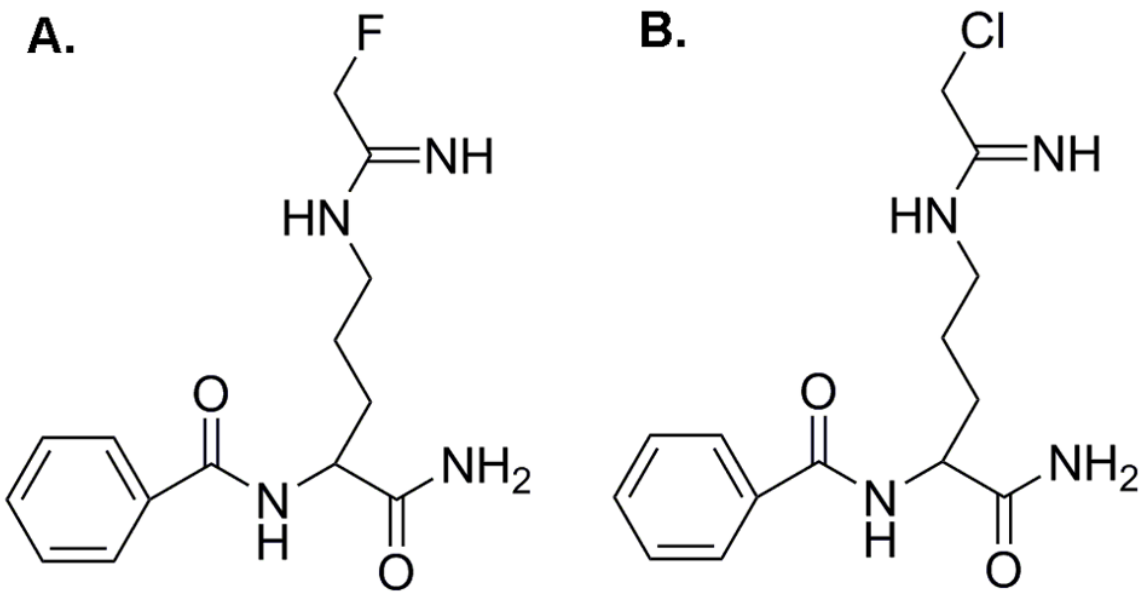

Figure 2.4. Structures of PAD inhibitors F-amidine and Cl-amidine. A) F-amidine and **B)** Cl-amidine share similar structures to arginine. Also these compounds are positive H bond donors that can sterically fit in the active site of PADs.

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CHAPTER 3

INHIBITING PROTEIN ARGININE DEIMINASES HAS ANTI-OXIDANT CONSEQUENCES*

ABSTRACT: Ulcerative colitis is a dynamic, idiopathic, chronic inflammatory condition that carries a high colon cancer risk. We previously showed that Cl-amidine, a small molecule inhibitor of the protein arginine deiminases (PADs), suppresses colitis in mice. Since colitis is defined as inflammation of the colon associated with infiltration of white blood cells that release free radicals, and citrullination is an inflammation-dependent process, we asked whether Cl-amidine has anti-oxidant properties. Here, we show that colitis, induced with azoxymethane (AOM) via intraperitoneal injection + 2% dextran sulfate sodium (DSS) in the drinking water, is suppressed by Cl-amidine (also given in the drinking water). iNOS, an inflammatory marker, was also down-regulated in macrophages by Cl-amidine. Since epithelial cell DNA damage associated with colitis is at least in part a result of an oxidative burst from overactive leukocytes, we tested the hypothesis that Cl-amidine can inhibit leukocyte activation, as well as subsequent target epithelial cell DNA damage *in vitro* and *in vivo*. Results are consistent with this hypothesis, and since DNA damage is a pro-cancerous mechanism, our data predicts that Cl-amidine will not only suppress colitis, but we hypothesize that it may prevent colon cancer associated with colitis.

* Erin E. Witalison, Xiangli Cui, Anne B. Hofseth, Venkataraman Subramanian, Corey P. Causey, Paul R. Thompson, and Lorne J. Hofseth. Journal of Pharmacology and Experimental Therapeutics. 2015; 353(1); 64-70. Reprinted with permission.

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3.1 INTRODUCTION

Ulcerative colitis is a heterogeneous, chronic, relapsing inflammatory bowel disease (IBD) that has a significant impact on quality of life. The millions of people who have this disease have an increased colon cancer risk. Despite varying causes (e.g. environmental factors, genetic susceptibility, imbalanced enteric bacteria), the end-result is an abnormal immune response with repeated episodes of colonic inflammation. While not everyone with colitis develops colon cancer, risk increases when disease duration exceeds 10 years, on the order of 0.5-1.0% annually (1). Conventional colitis treatments can reduce periods of active disease and help maintain remission, but these treatments often bring marginal results, patients become refractory, and develop serious side effects. Hence, we continue to look for less toxic and more efficacious drugs to suppress colitis and prevent colon cancer.

Protein Arginine Deiminases (PADs) are an enzyme family that converts peptidyl-Arginine to peptidyl-Citrulline (Arg→Cit) (2), a process called 'citrullination'. Mammals encode 5 isozymes within a single evolutionarily conserved gene cluster located on human chromosome 1 (1p35-36) (3). Mammalian PAD family members (PAD1-4 and 6) are highly related enzymes within and between individual species. PAD-mediated citrullination post-translationally modifies target proteins, which affects their function (3). Recently, protein citrullination has received increased attention due to its role in the pathogenesis of various inflammatory conditions, such as rheumatoid arthritis, multiple sclerosis, psoriasis, chronic obstructive pulmonary disease, neurodegenerative diseases and, due to its emerging role in various human and animal cancers (4). Biochemical and genetic evidence suggests that dysregulated PADs also contribute to the onset and

progression of colitis and colon cancer. For example, increased PAD levels are observed in colonic inflammatory lesions in Crohn's disease (5). We have confirmed that PADs are also overexpressed in the colons of colitis patients and in mouse colitis (6). PAD levels have also been reported to be increased in tumors, including colon adenocarcinomas (4, 7).

Importantly, we showed that Cl-amidine (Figure 3.1), a novel, small molecule PAD inhibitor, delivered by oral gavage, suppresses mouse colitis (6). It should be noted that Cl-amidine irreversibly inhibits PADs through the covalent modification of Cys645 in the active site of the enzymes (8); and due to steric hindrance, can only inhibit activated PADs that have undergone the calcium-dependent conformational change at the active site (9).

Because citrullination is inflammation-dependent (10), PADs cause citrullination, PAD inhibition by Cl-amidine suppresses inflammation, and inflammation is characterized by infiltrating leukocytes releasing free radicals ('oxidative stress') (11); it is possible that Cl-amidine also has anti-oxidant properties. To better understand the mechanisms by which Cl-amidine suppresses colitis, and potentially protects against colitis-associated colon cancer, we explored the anti-oxidant consequences of PAD inhibition as well as its ability to protect against DNA damage in target epithelial cells *in vitro* and *in vivo*.

3.2 Materials and Methods

3.2.1 Cl-amidine

The synthesis of Cl-amidine has been described previously (9, 12).

3.2.2 Cell culture and treatment

ANA-1 mouse macrophages or HCT116 human colon cancer cell lines were maintained in Dulbecco's Modified Eagles Media (DMEM, Hyclone, Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS, Biofluids, Rockville, MD), penicillin

(10 U/mL) and streptomycin (10 µg/mL, Biofluids, Rockville, MD). Experiments with Cl-amidine were carried out by pre-incubating cells with 0-10µg/mL (0-25 µM) Cl-amidine for 12 h. To activate ANA-1 cells, Cl-amidine was washed off, then cells were exposed to 100 U/mL IFN-γ (R&D Systems, Minneapolis, MN).

3.2.3 CO-CULTURE CONDITIONS

Co-culture experiments were carried out as previously described (13) with modifications. HCT116 colon cancer cells were seeded at 2.5 x 10^6 cells per 150 mm culture dish 24 h before exposure to Cl-amidine (10 µg/mL) for 12 h. Cl-amidine was washed off, then HCT116 cells were exposed to IFN-γ-activated or non-activated ANA-1 cells. ANA-1 cells were added to the actively growing colon cancer cells at a 3:1 ratio (ANA-1:HCT116 cells). The co-culture was incubated for 4 h before harvest. After harvest, HCT116 and ANA-1 cells were separated with MACS mini-column separators and $CD45^+$ micro-beads. HCT116 cells were examined for DNA damage by Comet analysis. ANA-1 cells were examined for oxidative bursts by chemiluminescence.

3.2.4 DSS MOUSE MODEL OF COLITIS

An outline of the colitis mouse model can be found in Figure 3.2A. For this, Dextran Sulfate Sodium (DSS; MP Biomedicals, Solon, OH; m.w. 36,000-50,000) mouse model, 40 eight week-old C57BL/6 male mice were injected with azoxymethane (AOM; 10 mg/kg i.p.) and divided into 4 groups. AOM was used because this experiment was carried out in parallel with a colon cancer study (using AOM/DSS); thus, we wanted to replicate each animal model as close as possible. Mice in Group 1 were given drinking water *ad libitum* throughout the experiment. One week after AOM injection, 2% DSS was added to the drinking water for Groups 2, 3, and 4. Groups 3 and 4 were given 0.25 mg/mL

and 0.5 mg/mL Cl-amidine, respectively, in the drinking water when 2% DSS was added to the water. Fresh doses of DSS and Cl-amidine were added to the drinking water every 48 h, ensuring compound stability throughout the experiment. Colitis induction was evaluated during this study by monitoring body weight changes, bloody and loose stool, and rectal bleeding with daily observations. We determined that colitis was being sufficiently induced in Group 2 as compared to Groups 1, 3, and 4 (data not shown). We did not examine colon cancer as an endpoint for these experiments because we wanted to determine whether Cl-amidine administered in the drinking water at our doses was effective in suppressing DSS-induced colitis before embarking on a lengthy, expensive mouse model of colitis-associated colon cancer.

0.5 mg/mL Cl-amidine is the equivalent to approximately 486 mg/day for humans. Our calculation of the human equivalent amount of Cl-amidine consumed by mice uses the body surface area normalization method (14), with the following assumptions: a typical mouse drinks 5 mL water daily and weighs 25 g; the average adult human weighs 60 kg. Therefore, 0.5 mg/mL x 5 mL drinking water/day = 2.5 mg Cl-amidine/day. An average mouse weighs 25 g, therefore 2.5 mg/25 g x 1000 g/1 kg = 100 mg/kg/day. As discussed by Reagan-Shaw (14), the human equivalent dose (HED, mg/kg) = animal dose (mg/kg) x [Animal Km/Human Km]. As such, HED (mg/kg) for mouse = 100 mg/kg x [3/37] = 8.1 mg/kg. If an average human adult weighs 60 kg, this equates to 8.1 mg/kg x 60 kg = 486 mg/day for humans. Correspondingly, 0.25 mg/mL is approximately the HED of 243 mg/day.

After 14 days, mice were euthanized. For 4 mice/group, colons were flushed out with 1x phosphate buffered saline (PBS), opened longitudinally then incubated in 10%

FBS/5mM Ethylenediaminetetraacetic acid (EDTA)/Ca^{2+}/Mg^{2+} free PBS for 15 min. Colons were shaken gently for 10 s and the single-cell suspension consisting of epithelial and inflammatory cells collected in the supernatant. Trypan blue staining revealed >95% viable cells by microscopic observation. Epithelial and inflammatory cells were separated using $CD45^+$ magnetic cell sorting technology, according to kit instructions (Miltenyi BioTec, Auburn, CA). Small aliquots of cells from each group were centrifuged, and a dry pellet was frozen at -80 ˚C for western blot analysis. $CD45^-$ epithelial cells were counted, equalized to 1 x 10^6 cells, centrifuged (1,500 rpm, 5 min). Pellets were resuspended in freezing media and frozen at -80 ˚C until Comet analysis. $CD45^+$ inflammatory cells were counted, equalized to 1 x 10^6 cells, centrifuged (1,500 rpm, 5 min.), and examined for oxidative bursts by chemiluminescence. For the remaining 6 mice/group, colons were removed and measured. Colons were cut longitudinally, Swiss-rolled, and fixed in 10% buffered formalin overnight, then processed for histopathology/immunohistochemistry by paraffin embedding and sectioning.

3.2.5 Quantification of inflammation to examine effects on colitis

Paraffin embedded tissues were serially sectioned, and one section from each mouse was stained with H&E. Sections were microscopically examined for histopathologic changes, as we have done previously (15).

3.2.6 Western blot analysis and antibodies

Western blots were carried out as described previously (16). Antibodies used include: iNOS (Rabbit polyclonal, diluted 1:1000; Cayman Chemicals, Ann Arbor, MI), Cox-2 (Rabbit polyclonal, diluted 1:2000; Cayman Chemicals, Ann Arbor, MI), SOD1 (Rabbit polyclonal, diluted 1:2000; Abcam, Cambridge, MA), GPx1 (Rabbit polyclonal, diluted

1:500; Abcam, Cambridge, MA), catalase (Rabbit polyclonal, diluted 1:500; Abcam, Cambridge, MA), and GAPDH (Rabbit monoclonal, clone 14C10, diluted 1:1000, Cell Signaling, Danvers, MA).

3.2.7 IMMUNOHISTOCHEMICAL STAINING

For immunohistochemical staining, serial sections of mouse colon tissues (processed as described above) were incubated with anti-iNOS (Rabbit Polyclonal, diluted 1:2000; Cayman Chemical, Ann Arbor, MI), catalase (Rabbit Polyclonal, diluted 1:1000; Abcam, Cambridge, MA), GPx1 (Rabbit Polyclonal, diluted 1:1000; Abcam, Cambridge, MA), and SOD1 (Rabbit Polyclonal, diluted 1:500; Abcam, Cambridge, MA). To ensure even staining and reproducibility, sections were incubated by slow rocking overnight in primary antibodies (4 °C) using the Antibody Amplifier™ (ProHisto, LLC, Columbia, SC). Sections were processed with EnVision+ System-HRP kit according to kit protocols (DakoCytomation, Carpinteria, CA). The positive control was colon cancer tissue and the negative control lacked primary antibody incubation. Immunohistochemistry was quantified as described previously (17).

3.2.8 MEASURING OXIDATIVE BURSTS

Oxidative bursts from inflammatory cells were measured by chemiluminescence according to kit directions (World Precision Instruments, Sarasota, FL); and described by our group previously (18).

3.2.9 COMET ANALYSIS

A Comet assay was performed according to kit instructions (CometAssay™, Trevigen, Gaithersburg, MD). Cells treated with hydrogen peroxide (200 μM, 20 min.) were positive controls. Fifty comets/treatment were captured and quantified with the

Automated Comet Assay Analysis System (Loats Associates, Inc., Westminster, MD). % DNA Damage is defined as the % DNA in the tail. % DNA in the tail is defined as: integrated tail intensity x 100/total integrated cell intensity = normalized measure of the % total cell DNA in the tail.

3.2.10 Statistical analysis

Mean differences between groups were compared by one-way ANOVA with Scheffe multiple comparison tests. The P-value chosen for significance in this study was 0.05.

3.3 Results

3.3.1 Cl-amidine delivered through the drinking suppresses DSS-induced colitis

We previously shown that Cl-amidine, delivered daily by oral gavage, suppresses DSS-induced colitis in mice (6). Because Cl-amidine has a relatively short half-life in mice (37 min) (19), we wanted to determine whether adding Cl-amidine in the drinking water would enable a slower delivery of similar dosages that had worked previously (6). In our previous study, mice were dosed with Cl-amidine at 75 mg/kg/day by oral gavage. Here, we estimate that 0.25 mg/mL and 0.5 mg/mL are equivalent to 50 mg/kg and 100 mg/kg daily (see calculations in Methods). Figure 3.2B shows that delivering Cl-amidine through the water effectively suppresses colitis induced by AOM+DSS. The addition of both 0.25 mg/mL and 0.5 mg/mL are equally effective, with the histology score nearly that of the AOM only control group. Similarly, Cl-amidine increased weight gain compared to AOM+DSS treated mice. It is important to note, that under such conditions, Cl-amidine does suppress protein citrullination (20).

Because mouse colon length shrinks with stress, inflammation, and ulceration, colon lengths were measured upon euthanasia. Compared with the AOM only group (Group 1: 8.7±0.15cm), the length was significantly reduced in the AOM+DSS group (Group 2: 7.7±0.27cm). Mice consuming AOM+DSS+0.25 mg/mL Cl-amidine (Group 3) had a statistically significant increase in colon length (9.0±0.15 cm) compared to the AOM+DSS group. Mice consuming AOM+DSS+0.5 mg/mL Cl-amidine (Group 4) had a statistically similar colon length (8.6±0.26 cm) to that of Groups 1 and 3, indicative of healthier, less inflamed colon in groups 1, 3, and 4.

To further verify the *in vivo* immunosuppressive effects of Cl-amidine on colon inflammation, we probed colon tissues for iNOS by immunohistochemistry. Figure 3.3 shows representative sections of stained tissues and quantification of iNOS. Overall, iNOS levels were elevated in AOM+DSS-treated mice, with staining appearing mostly in epithelial cells. iNOS staining was statistically significantly reduced in the AOM+DSS+Cl-amidine-treated mice. Such results both confirm and complement our H&E pathology results.

3.3.2 Cl-amidine inhibits inflammatory cell activation and associated DNA damage in target epithelial cells in vitro and in vivo.

Mucosal and DNA damage associated with colitis is, in part, a result of an oxidative burst from overactive inflammatory cells (21, 22). Since we found that pre-incubating ANA-1 mouse macrophages with Cl-amidine suppressed iNOS and Cox-2 induction by IFN-γ (Figure 3.4A), we tested the hypothesis that PAD inhibition by Cl-amidine can inhibit leukocyte activation with release of free radicals through an oxidative burst, and resultant epithelial cell DNA damage. Figure 3.4B shows that Cl-amidine (10 μg/mL)

inhibits oxidative bursts in cultured macrophages. Cells pretreated with Cl-amidine for 12 h have 60% less oxidative burst capacity than cells not treated with Cl-amidine. This indicates Cl-amidine blunts basal oxidative bursts. 1 h after activation with IFN-γ, Cl-amidine-treated cells have 60% of the oxidative burst capacity of untreated cells, indicating that Cl-amidine also protects from an induced oxidative burst. Thereafter, cells begin regaining their oxidative burst capacity, presumably because of the depletion of Cl-amidine.

Because an oxidative burst from macrophages can induce DNA damage in target epithelial cells, we co-cultured ANA-1 macrophages with HCT116 colon cancer cells (see Methods). Figure 3.4C shows a time-dependent increase in DNA damage, as assessed by Comet assay. Cells pre-incubated with Cl-amidine were significantly protected from DNA damage at 4 h after the initiation of co-incubation ($p < 0.01$).

We next asked whether Cl-amidine can activate anti-oxidant enzymes. Therefore, we measured the levels of key anti-oxidant enzymes, catalase, GPx1, and SOD1, which are shown to be reduced in models of IBD (23-25). Figure 3.5A shows that the level of each enzyme increases maximally when Cl-amidine is dosed at 5 μg/mL (12 μM) in ANA-1 murine macrophages. Also, we demonstrated that Cl-amidine treatment upregulates antioxidant enzymes in our mouse model of colitis as compared to AOM+DSS-treated mice (Figure 3.5B-D). As expected, the AOM only treated mice had low basal levels of catalase and GPx1 due to no induction of inflammation. Likewise, the AOM+DSS-treated mice had attenuated levels of catalase, GPx1, and SOD1 in the presence of significant inflammation. To note, the AOM only group did have a higher basal level of SOD1, however, SOD1 levels were restored upon Cl-amidine treatment (Figure 3.5D).

In order to test whether Cl-amidine inhibits an oxidative burst and associated DNA damage *in vivo,* isolated colon epithelial and inflammatory cells from mice treated with and without Cl-amidine (see Methods). Figure 3.6A shows that AOM+DSS-treated mice have increased levels of iNOS in $CD45^+$ and $CD45^-$ cells. iNOS induction is attenuated in mice consuming Cl-amidine. $CD45^+$ leukocytes were examined for an oxidative burst by chemiluminescence and we found that Cl-amidine suppresses CD45+ cell activity in the colon (Figure 3.6B). $CD45^-$ colon epithelial cells from the same mice were examined for DNA damage by Comet analysis. Figure 3.6C shows DNA damage is blunted in mice consuming Cl-amidine. Interestingly, but not surprisingly, there was appreciable DNA damage in the AOM+DSS group. Such damage is attributed to AOM, a carcinogen capable of causing DNA damage in the gastrointestinal tract (26, 27). These results indicate that Cl-amidine attenuates inflammatory cell activation and protects from colon epithelial cell damage *in vivo*. Such results provide mechanistic reasoning for the ability of Cl-amidine to attenuate colitis-associated mucosal damage, and the potential for protection against colon cancer development.

3.4 DISCUSSION

Here we have shown that the pan-PAD inhibitor, Cl-amidine, given to mice in drinking water, and shown by us to block protein citrullination in mice fed Cl-amidine (20), suppresses colitis (Figure 3.2). Additionally, we have shown that Cl-amidine suppresses an oxidative burst in leukocytes, and protects target epithelial cells from DNA damage *in vitro* (Figure 3.4) and *in vivo* (Figure 3.6). Increasing evidence shows that PADs are involved in the citrullination of multiple target proteins. Although there are some overlapping target proteins, each isozyme (PAD1-4 and PAD6) appears to target a unique

set of cellular proteins (28, 29). For instance, PAD4 (the most well-studied PAD isozyme) is involved in gene regulation and apoptosis, acting as a transcriptional co-regulator for p21, p53, p300, CIP1, ELK1, ING4, and nucleophosmin (30-34). Because Cl-amidine is a pan-PAD inhibitor (8), it is likely that it suppresses the citrullination of many of these PAD target proteins.

Because citrullination is an inflammation-dependent event (10), and we have previously shown (and show here) that Cl-amidine suppresses colon inflammation (Figure 3.2) (6), it is likely that citrullination of proteins specifically involved in colitis drives inflammation. Accordingly, we have shown here that PAD inhibition by Cl-amidine suppresses ROS release by inflammatory cells. This brings up the intriguing possibility that enzymes/proteins directly involved in ROS production are affected by citrullination. Such enzymes/proteins include not only TNF-α [a major target for treatment of IBD in humans that has been shown to be citrullinated, resulting in a change in activity (35)], but also downstream targets, such as iNOS and Cox-2; both of which were suppressed by Cl-amidine (Figures 3.3, 3.4 and 3.6). Our finding that catalase, GPx1 and SOD1 are all elevated by Cl-amidine *in vitro* and *in vivo* (Figure 3.5) is also consistent with the hypothesis that these anti-oxidant enzymes are mechanistically involved in the crossroads of Cl-amidine and its' ability to suppress an oxidative burst. Since Cl-amidine induces p53 (30, 36), and p53 drives the expression of anti-oxidant enzymes (37-42), potentially p53 repression by PADs (either by direct or upstream citrullination) is suppressing the ability of p53 to activate anti-oxidant enzymes. This hypothesis, though, would have to be tested.

In summary, we have shown that the pan-PAD inhibitor, Cl-amidine through the drinking water, is a viable treatment strategy for colitis. Our data reveal that Cl-amidine

directly inhibits leukocyte activation and target epithelial DNA damage within the colon. Due to the strong link between chronic DNA damage and increased cancer risk, we are carrying out separate, long-term studies to explore the hypothesis that Cl-amidine protects mice from inflammation-driven colon cancer. Further studies will also explore whether Cl-amidine works upstream of the colon in peripheral blood cells or lymphoid tissues. Indeed, other biological therapies can cause cellular apoptosis within spleens of treated mice (43). Here, we present the first line of evidence that a pan-PAD small molecule inhibitor (Cl-amidine) has anti-inflammatory properties in a colitis mouse model, with the ability to suppress leukocyte activation and prevent colon epithelial DNA damage both *in vitro* and *in vivo*.

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AUTHOR CONTRIBUTIONS

Participated in research design: Witalison, Cui, L.J. Hofseth, Subramanian, Thompson

Conducted experiments: Witalison, Cui, A.B. Hofseth

Contributed new reagents or analytic tools: Subramanian, Causey, Thompson

Performed data analysis: Witalison, Cui

Wrote or contributed to the writing of the manuscript: L.J. Hofseth, Witalison

FIGURES

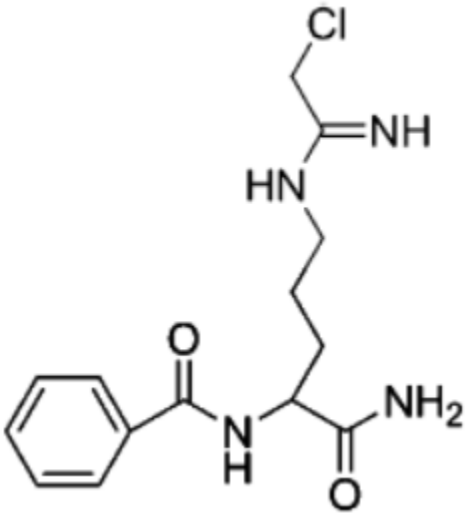

Figure 3.1. Structure of the pan-PAD inhibitor, Cl-amidine.

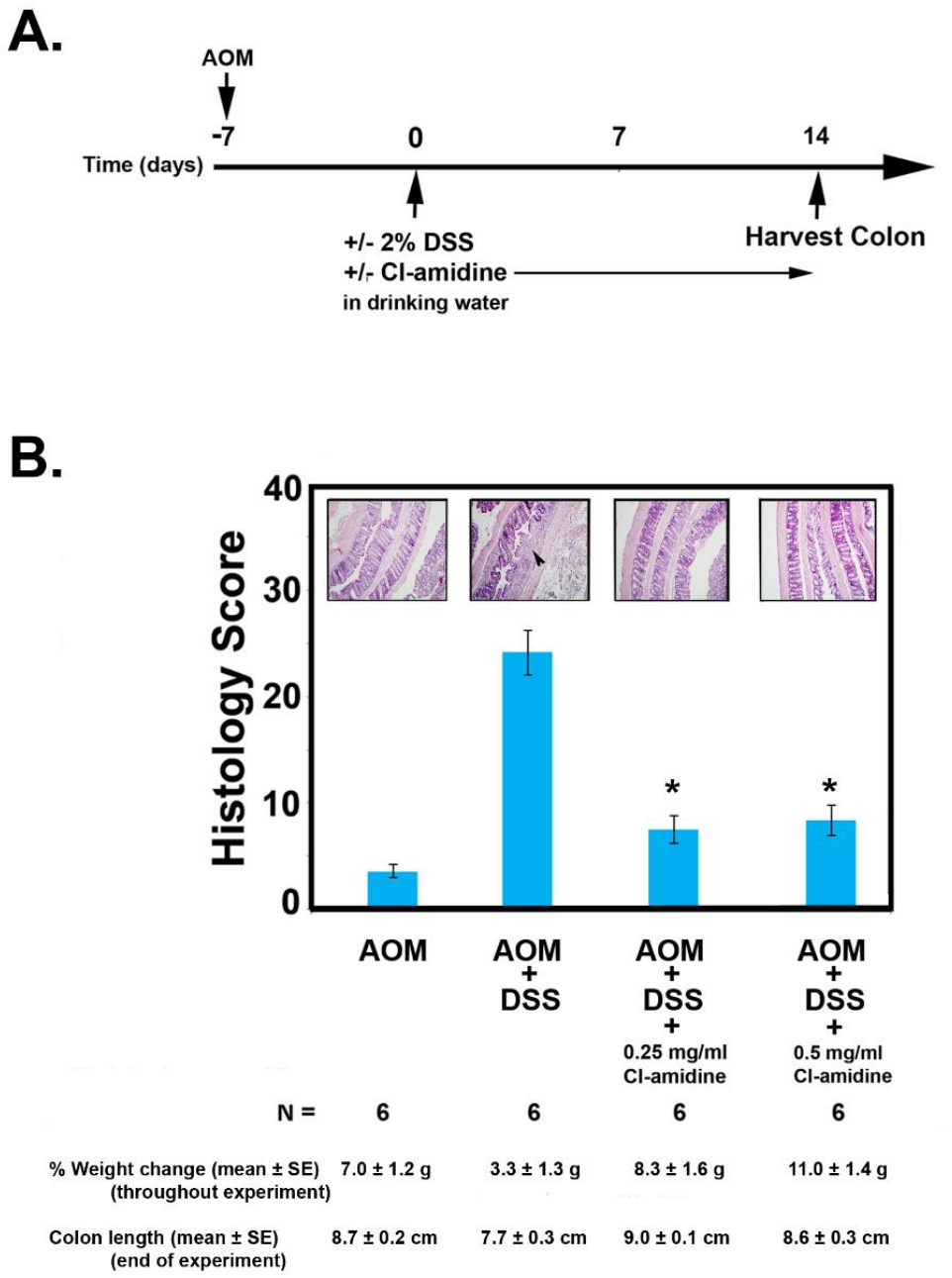

Figure 3.2. A) Outline of the AOM/DSS mouse model of colitis used in this study. **B)** Effects of Cl-amidine on the colon histology score in the acute AOM/DSS colitis model. Six mice from each group described in *Materials and Methods* were euthanized on day 14, and colons were measured and harvested. Then the histology score was determined. Weight was recorded every 48 hours during the 14-day experiment. Values represent the mean ± S.E. of the mean. Representative H&E-stained colons are shown for each group. *Significant difference from the AOM + DSS group ($P < 0.01$).

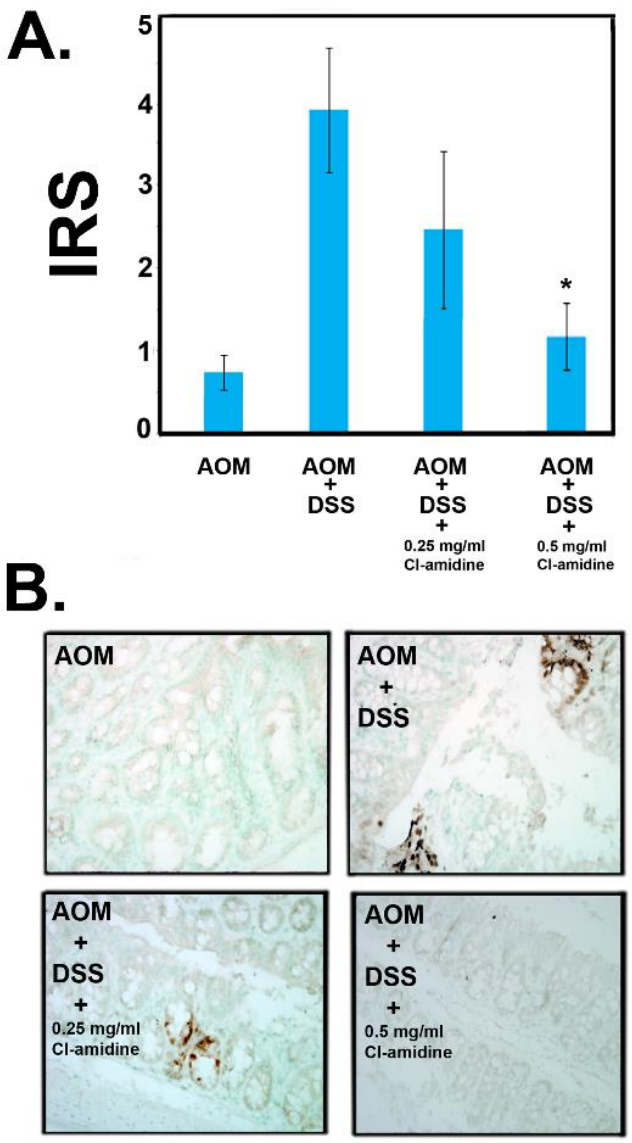

Figure 3.3. iNOS levels are reduced in the colons of mice treated with Cl-amidine. Six mice from each of the indicated groups were euthanized on day 14, and colons were harvested from each animal and stained with iNOS as described in *Materials and Methods*. **A)** Immunoreactivity score (IRS) for each group. Values represent the mean ± S.E. *Significant difference from the AOM + DSS only group ($P < 0.01$). **B)** Representative sections of indicated group. Positive staining is brown colored. (400x magnification).

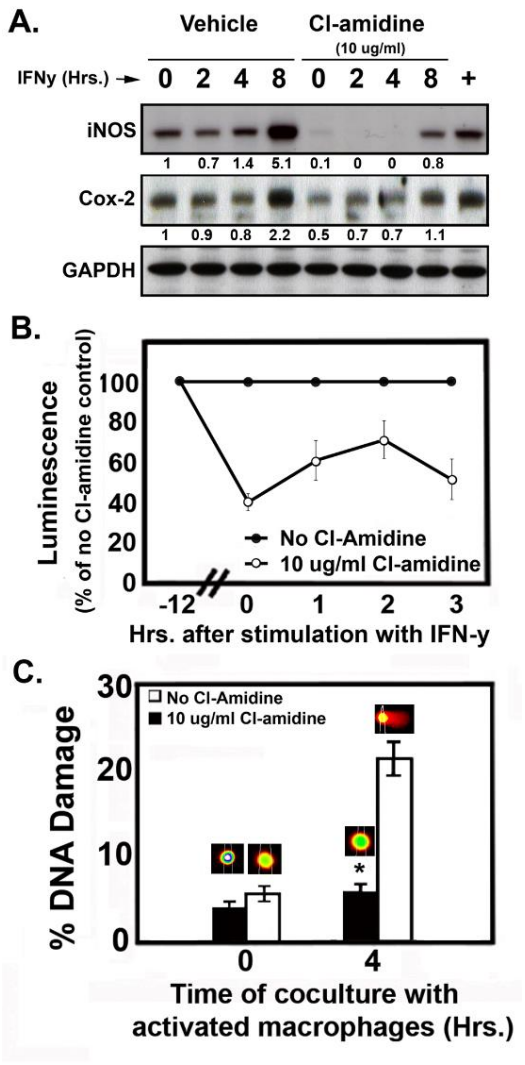

Figure 3.4. Cl-amidine attenuates the activation of macrophages and protects from DNA damage in target epithelial cells *in vitro*. A) iNOS and Cox-2 induction after treatment of ANA-1 mouse macrophages with IFN-γ. Numbers below each blot represent the GAPDH-adjusted density of each band, with the control (0 hour, no treatment) being a baseline of 1.0. The observation that for both markers (iNOS and Cox-2) density is lower in unstimulated cells exposed to Cl-amidine (0 hour, +10 µg/ml Cl-amidine, 5th lane) suggests Cl-amidine inhibits basal activity of macrophages. Accordingly, it also inhibits the activation of macrophages. **B)** An oxidative burst in ANA-1 mouse macrophages is attenuated by pretreatment with Cl-amidine (10 µg/ml). Chemiluminescence was measured as described in *Materials and Methods*. Results were compared with no Cl-amidine control (± S.E.). **C)** In the presence of an oxidative burst, target epithelial cells (HCT116 colon cancer cells) pretreated with 10 µg/ml Cl-amidine are protected from DNA damage. Results are represented as the mean Comet tail moment ± S.E., scoring 50 comets/treatment group. Representative images of Comets in each treatment group are shown above each bar graph. *Significant difference from the untreated (No Cl-amidine) macrophages that were cocultured for 4 hours ($P < 0.01$).

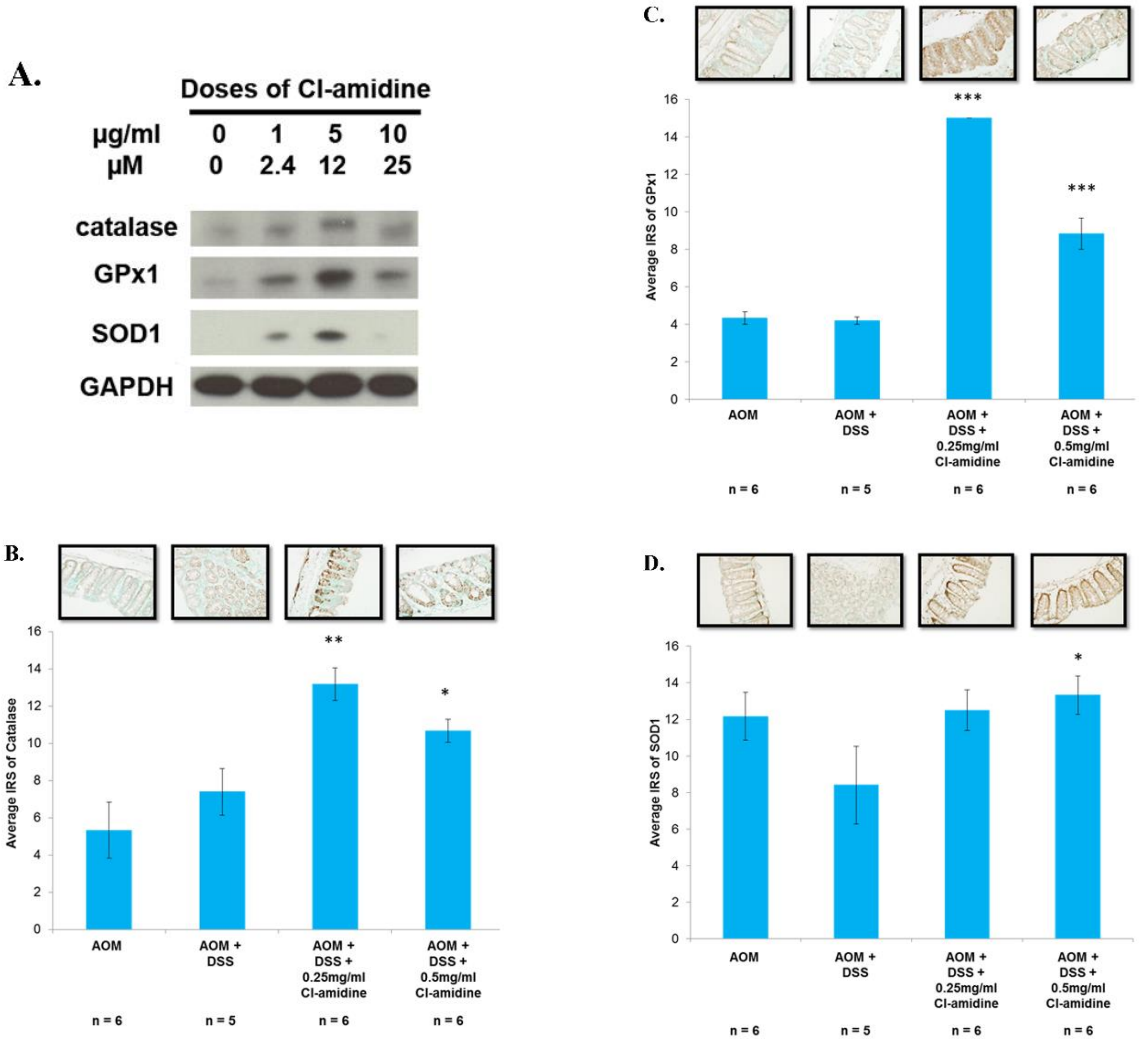

Figure 3.5. Cl-amidine induces antioxidant enzymes (catalase, GPx1, SOD1) in IFN-γ–stimulated ANA-1 mouse macrophages and *in vivo*. A) ANA-1 mouse macrophage cells were pretreated with 0–10 µg/ml Cl-amidine for 12 hours and then cells were stimulated with IFN-γ for 8 hours. Antioxidant enzymes of interest were suppressed in activated cells pretreated with 10 µg/ml (lane 4). **B–D)** Mice from each of the indicated groups were euthanized on day 14, and colons were harvested from each animal and stained with catalase **(B)**, GPx1 **(C)**, and SOD1 **(D)** as described in *Materials and Methods*. Immunoreactivity scores (IRS) are shown for each group. Values represent the mean ± S.E. Significance is compared with the AOM + DSS only group: **P* < 0.05; ***P* < 0.01; ****P* < 0.005. Representative sections of each group were taken at 400x magnification, and positive staining is brown colored.

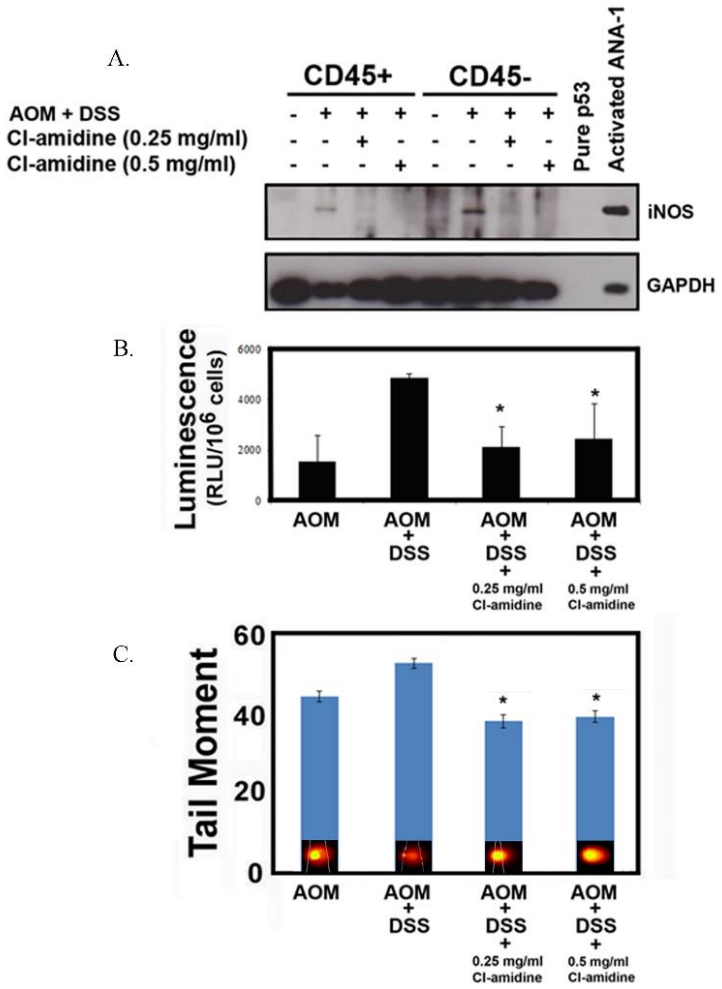

Figure 3.6. Cl-amidine attenuates the activation of white blood cells and protects from DNA damage in target epithelial cells *in vivo*. Mice were injected with AOM (10 mg/kg), then 1 week later they were given either water ad libitum or 2% DSS in the drinking water for 14 days as described in *Materials and Methods* and in Figure 3.2. **A)** Protein lysates from scraped mucosa of the colon (4 mice per group; lysates were combined) were examined for iNOS and GAPDH (internal control). Mice consuming 2% DSS had activation of only iNOS in both $CD45^+$ and $CD45^-$ cells. Mice consuming Cl-amidine + 2% DSS had iNOS attenuated in both cell types. **B)** After column separation of inflammatory cells from mucosal cells, we examined an oxidative burst of $CD45^+$ inflammatory cells (4 mice per group). Mice consuming Cl-amidine + DSS exhibit $CD45^+$ inflammatory cells with attenuated activity compared with mice on 2% DSS only. Chemiluminescence was measured as described in *Materials and Methods* and expressed as mean (± S.E.) relative light units (RLU) per 1 x 10^6 cells. *Significant difference from the AOM + DSS–only group ($P < 0.05$). **C)** Mucosal epithelial cells were examined for DNA damage by Comet analysis. Results are presented as the mean (± S.E.) tail moment from 200 Comets taken from 4 mice per group. *Significant difference from the AOM + DSS–only group ($P < 0.01$). Representative Comets for each group are shown.

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CHAPTER 4

MOLECULAR TARGETING OF PROTEIN ARGININE DEIMINASES TO SUPPRESS COLITIS AND PREVENT COLON CANCER*

ABSTRACT: Ulcerative colitis (UC) is a chronic disease, in which the lining of the colon becomes inflamed and develops ulcers leading to abdominal pain, diarrhea, and rectal bleeding. The extent of these symptoms depends on disease severity. The protein arginine deiminase (PAD) family of enzymes converts peptidyl-Arginine to peptidyl-Citrulline through citrullination. PADs are dysregulated, with abnormal citrullination in many diseases, including UC and colorectal cancer (CRC). We have developed the small molecule, pan-PAD inhibitor, Chlor-amidine (Cl-amidine), with multiple goals, including treating UC and preventing CRC. Building off our recent results showing that: 1) Cl-amidine suppresses colitis *in vivo* in a dextran sulfate sodium (DSS) mouse model; and 2) Cl-amidine induces microRNA (miR)-16 *in vitro* causing cell cycle arrest, we tested the hypothesis that Cl-amidine can prevent tumorigenesis and that miR-16 induction, by Cl-amidine, may be involved *in vivo*. Consistent with our hypothesis, we present evidence that Cl-amidine, delivered in the drinking water, prevents colon tumorigenesis in our mouse model of colitis-associated CRC where mice are given carcinogenic azoxymethane (AOM), followed by multiple cycles of 2% DSS to induce colitis. To begin identifying mechanisms, we examined the effects of Cl-amidine on miR-16. Results show miR-16

* Erin E. Witalison, Xiangli Cui, Corey P. Causey, Paul R. Thompson, and Lorne J. Hofseth. Oncotarget. 2015; 6(34); 36053-36062. Reprinted with permission.

suppression during the colitis-to-cancer sequence in colon epithelial cells, which was rescued by drinking Cl-amidine. Likewise, Ki67 and cellular proliferation targets of miR-16 (Cyclins D1 and E1) were suppressed by Cl-amidine. The decrease in cell proliferation markers and increase in tumor suppressor miRNA expression potentially define a mechanism of how Cl-amidine is suppressing tumorigenesis *in vivo*.

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4.1 INTRODUCTION

Ulcerative colitis (UC) is a chronic, relapsing inflammatory bowel disease that affects millions of people worldwide and causes symptoms of abdominal pain, diarrhea, and rectal bleeding. Due to the chronic inflammatory state of UC, which causes cyclical oxidative stress and leads to DNA damage, patients with UC are at a higher risk of developing colorectal cancer (CRC) (1). The probability of developing CRC increases yearly once UC develops, with up to an approximate 20% incidence after 30 years (1, 2). Current treatment options help to treat the symptoms, prevent flares, and heal the damaged colon; however, the treatment outcomes are often marginal, patients become refractory, and there are dangerous adverse side effects. Therefore, we continue to investigate novel therapies showing reduced toxicity and more substantial efficacy in treating UC and preventing CRC.

The protein arginine deiminase (PAD) family of enzymes converts peptidyl-Arginine to peptidyl-Citrulline through a process called 'citrullination' (3). There are five isozymes found in mammals (PAD1-4 and 6), with 70-95% homology in their amino acid sequence (4). The PAD isozymes are encoded by a cluster of homologous genes found on Chromosome 1 in humans (4, 5). Citrullination can affect protein-protein interactions, hydrogen bond formation, and protein structure due to the shift from the positively charged peptidyl-Arginine to the neutral peptidyl-Citrulline (5). At physiological activity levels, PADs regulate many cell signaling pathways including differentiation, apoptosis, and gene transcription (6). However, over the past decade, it is becoming increasingly apparent that aberrant PAD activity is involved in many human inflammatory diseases such as: rheumatoid arthritis, Alzheimer's disease, and multiple sclerosis (3, 7-10). Concerning our

study, PADs, especially PAD4, are found to be dysregulated in UC and CRC (11-14). In UC and other chronic inflammatory, autoimmune diseases, it is thought that the accumulation of citrullinated proteins is what leads to an abnormal immune response that further exacerbates the inflammatory response (6).

We have developed a novel, small molecule inhibitor of PADs, named N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide or Chlor-amidine (Cl-amidine). Cl-amidine prevents citrullination by covalently modifying a conserved cysteine residue in the active site of the PADs, causing irreversible inactivation of the enzyme (6). Cl-amidine is an excellent candidate drug for our CRC prevention studies because it: 1) exhibits little toxicity at doses we use here *in vitro* and *in vivo*; 2) displays no immunosuppressive effects in multiple disease models associated with PAD dysregulation; 3) effectively suppresses colitis in mice when given by oral gavage; and 4) increases tumor suppressor microRNA (miRNA) levels *in vitro* (14-17). Based on these precedents, we tested the hypothesis that Cl-amidine can prevent tumorigenesis in the azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model of colitis-associated CRC. Mechanistic insight is also gained by examining the levels of miRNA changes during this process.

4.2 Results

4.2.1 Cl-amidine suppresses AOM/DSS-induced tumorigenesis in mice

To ensure that 2% DSS was effectively causing colitis in the treated mice, we determined the histology score of four euthanized mice (per group) at *day 35*. In Figure 4.1B, histology scores for ulceration and inflammation indicate that the AOM + DSS only group does have a significantly higher score than the AOM only group. As expected at

this time point, despite the varying severity of colitis in the treatment groups, none of the mice had developed tumors.

At *day 70*, mice were euthanized, and we recorded the number mice with tumors (Table 4.1) and the average number of tumors per mouse in each group (Figure 4.2A; tumor multiplicity). We also measured the size of each tumor (Table 4.1) and captured representative images of the methylene blue stained colons (Figure 4.2B). No tumors were found in the control AOM only mice and, as predicted, the AOM + DSS only group (Group 2: 2.8 ± 0.68 tumors/animal) had the highest tumor multiplicity. Mice treated with Cl-amidine (Group 3: 0.07 ± 0.07 tumors/animal; Group 4: 0.67 ± 0.23 tumors/animal) had significantly reduced tumor multiplicity than the AOM + DSS only group. Interestingly, there was a significant difference in tumor multiplicity ($p<0.05$) between the Cl-amidine treated groups where the higher dosage group (Group 4: 0.25 mg/mL Cl-amidine) had a higher tumor multiplicity than the lower dosage group (Group 3: 0.05 mg/mL Cl-amidine). Although at this point we are unable to explain this observation, perhaps a certain basal level of citrullination is necessary to carry out functions, such as apoptosis. Since the citrullination of proteins involved in apoptosis (e.g. vimentin, nucleophosmin, nuclear lamin C) facilitates the process of apoptosis (18-21), the higher dose of Cl-amidine may be inhibiting the citrullination necessary for the progression of apoptosis and aiding in the development of tumors, thus accounting for the increased tumor incidence. Nevertheless, the overall results from this model show for the first time that Cl-amidine is capable of preventing tumorigenesis associated with chronic colitis.

4.2.2 Cl-amidine increases miR-16 expression and downregulates cell proliferative miR-16 targets in mice

We have previously shown that Cl-amidine increases miR-16 expression in a p53-dependent manner resulting in a cell cycle arrest *in vitro* (17). Since the inhibition of cell proliferation is a goal of many anti-cancer drug therapies, we hypothesized that Cl-amidine is preventing tumorigenesis by increasing miR-16 expression *in vivo*. To test this, we measured miR-16 expression levels in isolated colon epithelial (CD45-) cells at *day 35* (see methods). We chose to investigate the miR-16 expression levels at the *day 35* time point because we were interested in the mechanism preventing tumorigenesis at *day 70*. Figure 4.3 shows the relative fold change in miR-16 levels in the epithelial cells. Consistent with our previously published *in vitro* data (17) and our current hypothesis, miR-16 expression in the AOM + DSS only group was significantly lower than the AOM only group and both Cl-amidine treated groups. Furthermore, the lower level of miR-16 expression in epithelial cells from the higher dosage group (0.25 mg/mL Cl-amidine), compared to the lower dosage group (0.05 mg/mL Cl-amidine), is highly suggestive of a direct correlation between the expression level of miR-16 and tumor incidence; however, the cause of this variability between Cl-amidine treatment groups is currently unknown.

miR-16 has multiple cell proliferation targets, such as Cyclin D1 and Cyclin E1; supporting the premise that it is a tumor suppressor miRNA (17, 22-25). If miR-16 expression is increased with Cl-amidine treatment (Figure 4.3), then we expect to see the downregulation of these cell proliferation targets of miR-16. Indeed, we confirmed that protein expression of Cyclins D1 and E1 was suppressed in the Cl-amidine treated groups when compared to the AOM + DSS only group (Figures 4.4A and B). To further verify the

repression of cell proliferation in the mice treated with Cl-amidine, we performed IHC staining for the cell proliferation marker, Ki67, in colons collected at *day 35* (Figure 4.4C). In the colons collected at *day 35*, the IRS of the AOM + DSS only groups were significantly higher than the control AOM only groups (Figure 4.4A-C). For Cyclin D1 and Ki-67 stained colon sections, the lower dose Cl-amidine group (0.05 mg/mL) had a lower IRS than the AOM + DSS only group; however, the group receiving 0.25 mg/mL Cl-amidine was not significantly lower. Interestingly, the IRS values of Cyclin D1 and Ki67 staining at *day 35* revealed a similar trend to the tumor incidence and miR-16 expression levels in colon epithelial cells. For Cyclin E1 stained colon sections, the groups treated with Cl-amidine both had lower IRS values than the AOM + DSS only group, but the lower dose Cl-amidine group (0.05 mg/mL) was not significantly less. These results are consistent with the hypothesis that Cl-amidine is suppressing tumorigenesis in our mouse model by inhibiting cell proliferation via increased miR-16 expression.

4.3 DISCUSSION

In this study, we provide evidence that Cl-amidine, a small molecule inhibitor of PADs, administered to mice in drinking water suppresses colitis (Figure 4.1) and tumorigenesis (Figure 4.2; Table 4.1). To uncover the mechanism by which Cl-amidine is acting, we revealed that Cl-amidine increases miR-16 expression in colon epithelial cells (Figure 4.3). Likewise, Cl-amidine treatment decreases protein expression of the miR-16 targets, Cyclins D1 and E1, and the cell proliferation marker, Ki67 (Figure 4.4). Figure 4.5 depicts the unique mechanism by which Cl-amidine prevents the tumorigenesis of CRC in our mouse model.

PADs are calcium-dependent. Therefore, they are usually inactive under physiological levels (10^{-8} to 10^{-6} M); and only activating during certain events (i.e. apoptosis and terminal epidermal differentiation) where calcium levels are above physiological concentrations (5, 18). Interestingly, and important to this study, patients with active Crohn's disease or UC have moderately higher Ca^{2+} levels than healthy controls (26). Not surprisingly, then, citrullination (the post-translationally modified product of active PADs) is elevated in colitis (27). During apoptosis and terminal epidermal differentiation, PADs are found to citrullinate structural proteins, such as vimentin and filaggrin, causing partial unfolding of the proteins (5, 18). The citrullination of these structural proteins promotes the overall degradation of the cells during cell death. PADs, specifically PAD4, have also been found to regulate gene expression (28-33). Many of the genes that the PADs regulate are involved in the progression of the p53 pathway, including ING4, p300, and HDAC2 (30-32). With the involvement of PADs in pathways that are crucial for proper cell growth and cell death, it is readily apparent that aberrant PAD activity can lead to deleterious consequences (e.g. abnormal DNA damage repair response, protein misfolding, protein inactivation).

Additionally, citrullination controls the expression of tumor suppressing miRNAs. miRNAs are regulators of genes that control various cell signaling processes, such as cell proliferation, apoptosis and stress response (34). As a result of an inflammatory response, such as that of UC, miRNAs can be altered and expression levels can fluctuate (35). Although other miRNAs have been found associated with either mouse or human UC, we focused here on miR-16 because our previous *in vitro* data showed that Cl-amidine induces miR-16 expression and decreases the expression of several miR-16 targets (i.e. Cyclins D1,

D2, D3, E1, and CDK6) involved in the progression through the cell cycle (17). Likewise, miR-16 is found at lower levels in CRC than in normal tissue (36). One prospective pathway through which Cl-amidine may be upregulating miR-16 expression levels involves the tumor suppressor p53. p53 boosts the post-transcriptional maturation of miR-16 and Cl-amidine is found to increase miR-16 expression in a p53-dependent manner *in vitro* (17, 37-39).

In summary, we have shown that Cl-amidine suppresses colitis and, in this current study, suppresses tumorigenesis in mice given Cl-amidine dosages in drinking water (14). Our current *in vivo* study is a substantial extension of our previous mechanistic *in vitro* data and suggests that increased miR-16 expression in mice treated with Cl-amidine results in decreased tumor formation (17). When miR-16 is downregulated, this relieves its inhibition of cell proliferative targets, like Cyclin D1 and E1. Because our results demonstrate that Cl-amidine can suppress these targets of miR-16, this verifies the activity of miR-16 throughout our model. These findings display a significant development in our knowledge of *in vivo* mechanisms by which PAD inhibition can suppress colon cancer. Future studies will explore the mechanism(s) by which Cl-amidine is increasing miR-16 expression and will determine optimum dosages for preventing tumorigenesis. Overall, this study presents Cl-amidine as a viable cancer preventative therapy against colitis-associated colorectal cancer and provides an innovative mechanism of action involving the upregulation of miR-16, ultimately leading to decreased cell proliferation and prevention of tumorigenesis *in vivo*.

4.4 Materials and Methods

4.4.1 Cl-amidine

The synthesis of Cl-amidine has previously been described (6, 40).

4.4.2 AOM/DSS mouse model of colitis-associated colorectal cancer

This mouse model of colorectal cancer is outlined in Figure 4.1A. C57BL/6 male mice (8-12 weeks old; Jackson Laboratory) were used in accordance with protocols approved by the Institutional Animal Care and Use Committees of University of South Carolina. AOM (10 mg/kg) was injected into each mouse via intraperitoneal injection. The mice were divided into four groups where Group 1 received drinking water *ad libitum* throughout the experiment. One week after AOM injection, Groups 2-4 began cycles of 2% DSS (MP Biomedicals, Solon, OH; m.w. 36 000 -50 000) given in the drinking water for 70 days. Groups 2-4 were subjected to three cycles, each consisting of one week with 2% DSS in the water followed by two weeks of normal drinking water. The DSS cycles were aimed to simulate the active inflammatory and remission states of UC. Groups 3 and 4 were administered 0.05 mg/mL and 0.25 mg/mL Cl-amidine, respectively, daily in the drinking water beginning at *day 14* and ending at *day 70*.

0.05 mg/mL and 0.25 mg/mL, which is the equivalent of 10 mg/kg/day and 50 mg/kg/day per mouse, respectively; or the human daily equivalent of 48.6 mg and 243 mg, respectively. Calculations are as follows: assuming an average mouse weighs 25 g and drinks approximately 5 mL per day, then 0.25 mg / 25 g x 1000 g / 1 kg = 10 mg/kg daily. The human equivalent dose (HED, mg/kg) = animal dose (mg/kg) x [Animal Km / Human Km] (41). The HED (mg/kg) for mouse = 10 mg/kg x [3/37] = 0.81 mg/kg. Therefore, assuming an adult human weighs approximately 60 kg on average, the human equivalent

is 0.81 mg/kg x 60 kg = 48.6 mg daily. Using the same equations, the 0.25 mg/mL mouse dosage is approximately the equivalent of 243 mg daily for humans. We chose these doses because our previous work has shown that 5 - 75 mg/kg/mouse per day was effective in a dose-responsive manner at suppressing DSS-induced colitis in mice (14). Since 5 mg/kg/day was only modestly effective, and 75 mg/kg/day is the Maximal Tolerable Dose, we wanted to investigate the potency of multiple doses within this range (14, 15). Thus we chose 0.05 mg/mL and 0.25 mg/mL delivered in the drinking water. We reasoned Cl-amidine would work better delivered in the drinking water because Cl-amidine has a short half-life *in vivo* (15). Therefore delivering the same amount throughout the day over a long period time (in water) versus a bolus amount (oral gavage) would be a better method with short-life compounds. We also determined that Cl-amidine is stable in DSS-spiked water for 48 h (data not shown) and the mice drink approximately the same amount of water daily regardless of treatment (Figure 4.6).

At *day 35*, after 1.5 cycles of DSS, 10 mice from each group (1-4) were euthanized to ensure that the DSS was effective and for further analysis. Colons from each mouse were removed, cut longitudinally, and washed with 1x phosphate buffered saline (PBS). The colons from four mice from each group were Swiss-rolled, fixed in 10% buffered formalin overnight, paraffin embedded, and then sectioned for histopathology/immunohistochemistry. The colons from the remaining six mice/group were incubated in 10% fetal calf serum/5 mM ethylenediaminetetraacetic acid (EDTA)/Ca^{2+}/Mg^{2+} free PBS for 15 min. Colons were then shaken gently for 10 sec and the single cell suspension, consisting of epithelial and inflammatory cells, collected in the supernatant. Inflammatory cells were separated from epithelial cells using CD45+

magnetic microbeads that select for the inflammatory cells, according to kit instructions (mouse $CD45^+$ MicroBeads, Miltenyi Biotec, Auburn, CA). These separated cells were immediately processed for miRNA analysis to avoid any breakdown of the RNA integrity.

At *day 70*, the remaining 15 mice from each of the groups were euthanized. Colons were removed, cut longitudinally, and washed with 1x PBS. Tumor incidence and size were recorded for each colon. Colons from each group were processed for histopathology/immunohistochemistry in the same manner as the colons harvested at *day 35*.

4.4.3 Quantification of inflammation

Colons prepared for histopathology were stained with hematoxylin and eosin (H&E). Sectioned H&E stained samples were examined microscopically and scored as previously described (14). Briefly, the histology score for a sample was determined by adding the scores for inflammation severity, inflammation extent, and crypt damage; then, multiplying by the score for percent area involvement. The inflammation severity was scored on a scale of 0-3: 0 (no inflammation), 1 (minimal), 2 (moderate), and 3 (severe). Inflammation extent was scored on a scale of 0-3: 0 (no inflammation), 1 (mucosa only), 2 (mucosa and submucosa), and 3 (transmural). Crypt damage was scored on a scale of 0-4: 0 (no crypt damage), 1 (one-third of crypt damaged), 2 (two-thirds damaged), 3 (crypts lost and surface epithelium intact), and 4 (crypts lost and surface epithelium lost). Percent area involvement was scored on a scale of 0-4: 0 (0% involvement), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The minimum possible score is 0 and the maximum possible score is 40.

4.4.4 MiR-16 EXPRESSION

Total miRNA was extracted from separated inflammatory (CD45+) and epithelial (CD45-) cells according to miRNeasy Mini Kit instructions (Qiagen). RNA concentration and stability were measured by the Nanodrop 2000 (Nanodrop, Wilmington, DE). 10 ng of total RNA were used to make cDNA using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to kit instructions. We also used hsa-miRNA-16 primers for miR-16 detection and small nuclear protein RNU6B (U6) for normalization (Applied Biosystems, Foster City, CA). Quantitative Real-Time PCR to measure miR-16 and U6 was performed using the TaqMan miRNA Assay (Applied Biosystems, Foster City, CA) with the 7300 PCR Assay System (Applied Biosystems, Foster City, CA). The relative fold change (FC) of miR-16 expression, as compared to U6 expression, was determined based on the comparative threshold cycles (Ct) of miR-16 and U6. All samples were analyzed in triplicate.

4.4.5 IMMUNOHISTOCHEMICAL STAINING

Paraffin-embedded sections of mouse colonic tissue were incubated (4˚C) in Cyclin D1 (Rabbit monoclonal, cat# TA307019, diluted 1:100, Origene, Rockville, MD), Cyclin E1 (rabbit polyclonal, cat# TA311853, diluted 1:1000, Origene, Rockville, MD), and anti-Ki67 (Rabbit polyclonal, cat# PA5-19462, diluted 1:5000, Pierce, Rockford, IL) primary antibodies by slow rocking overnight with Antibody Amplifier (ProHisto, Columbia, SC) to ensure even and reproducible staining. Samples were then processed using the EnVision+ System HRP kits (DAKO, Carpinteria, CA) according to kit instructions. The chromagen, diaminobenzidine, was added and then the samples were counterstained with methyl green. Stained tissues were objectively scored based on two criteria: 1) percentage

of tissue stained, and 2) the staining intensity. The percentage of tissue stained was scored on a scale of 0-5: 0 (0% positive staining), 1 (<10%), 2 (11-25%), 3 (26%-50%), 4 (51%-80%), or 5 (>80%). Staining intensity was scored on a scale of 0-3: 0 (Negative staining), 1 (Weak), 2 (Moderate), or 3 (Strong). Scores from each criteria were multiplied to get the final immunoreactivity score (IRS).

4.4.6 STATISTICAL ANALYSIS

Mean differences between groups were compared by one-way ANOVA with Scheffe multiple comparison tests. The p-value chosen for significance in this study was 0.05.

Conflict of Interest Statement

PRT is a cofounder and consultant to Padlock Therapeutics.

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TABLES

Table 4.1. Tumor incidence, multiplicity, and size are reduced in the colons of mice treated with Cl-amidine. Tumor incidence (the number of mice per group with tumors) is shown alongside the percentage of mice with tumors. The average number of tumors per mouse (tumor multiplicity) is shown and is also divided based on size (<1 mm and >1 mm at the largest dimension). Tumor multiplicity based on tumor size is given as mean ± standard error of the mean (SE). Significant differences from the AOM + DSS only group are indicated by * ($p<0.05$) and *** ($p<0.005$).

	Tumor Incidence	**Average # of tumors**	**Average tumor multiplicity <1mm**	**Average tumor multiplicity >1mm**
AOM	0 (0%)	0	0 ± 0*	0 ± 0***
AOM + DSS	13 (86.7%)	2.80	0.67 ± 0.37	2.13 ± 0.53
AOM + DSS + 0.05mg/ml Cl-amidine	1 (7.1%)	0.07	0 ± 0*	0.07 ± 0.07***
AOM + DSS + 0.25mg/ml Cl-amidine	7 (46.7%)	0.67	0 ± 0*	0.67 ± 0.23*

FIGURES

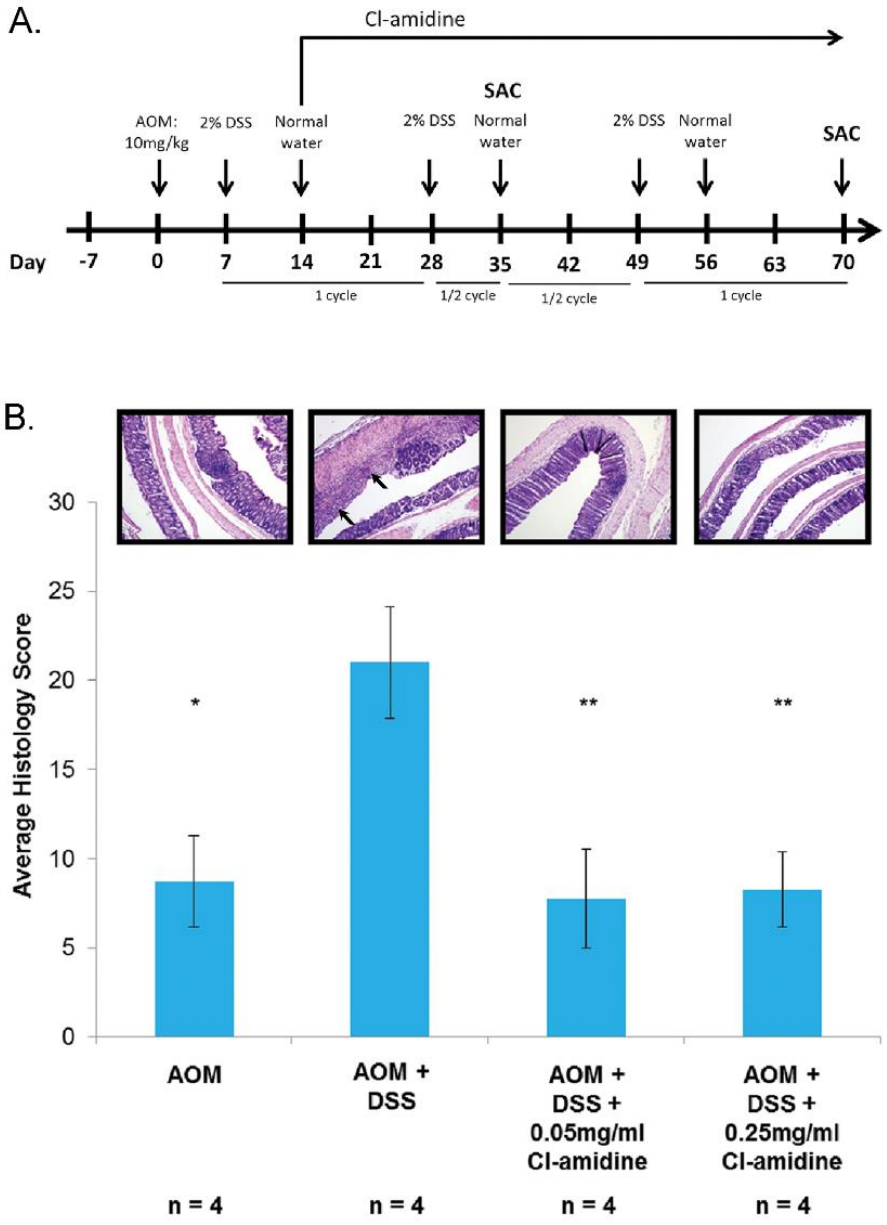

Figure 4.1. A) Outline of the AOM/DSS mouse model of colorectal cancer used in this study. **B)** Histology scoring of colon tissue samples from the AOM/DSS model of colon cancer at *35 days*. Mice from each group were euthanized at *day 35* and *day 70* as described in the methods. Colons were harvested and processed to determine the histology score at *day 35*. Values represent the mean ± SE. Representative H&E stained colon sections (100x magnification) are shown for each group. Arrows indicate areas of inflammation. Significant differences from the AOM + DSS only group are indicated by * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.005$).

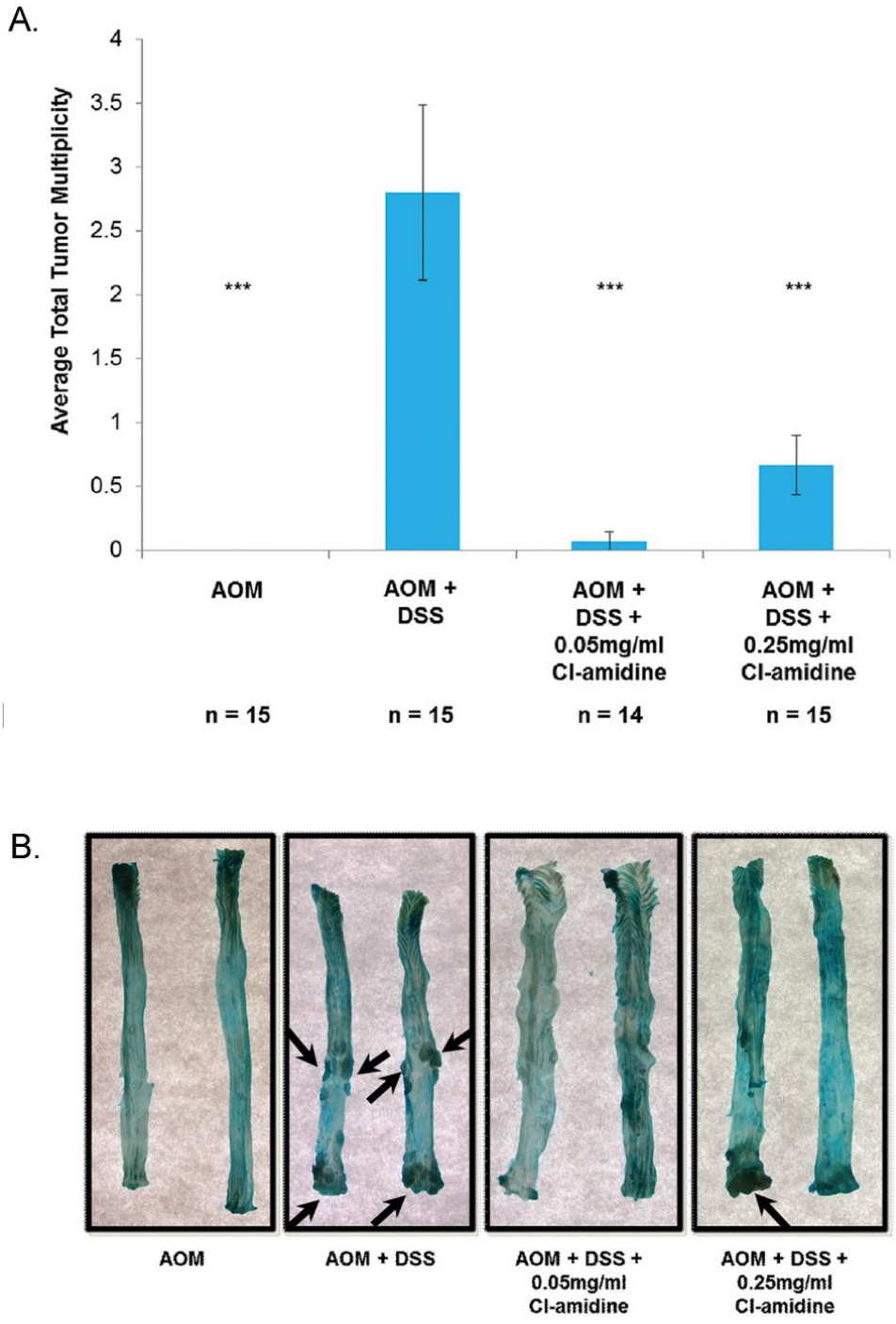

Figure 4.2. Tumor multiplicity is reduced in the colons of mice treated with Cl-amidine. Mice from each group were euthanized at *day 70* as described in the methods. Colons were removed and cut longitudinally. **A)** Tumor multiplicity in the AOM/DSS model of colon cancer at *70 days*. Significant differences from the AOM + DSS only group are indicated by *** ($p<0.005$). **B)** Representative methylene blue stained colons are shown for each group. Arrows indicate tumors.

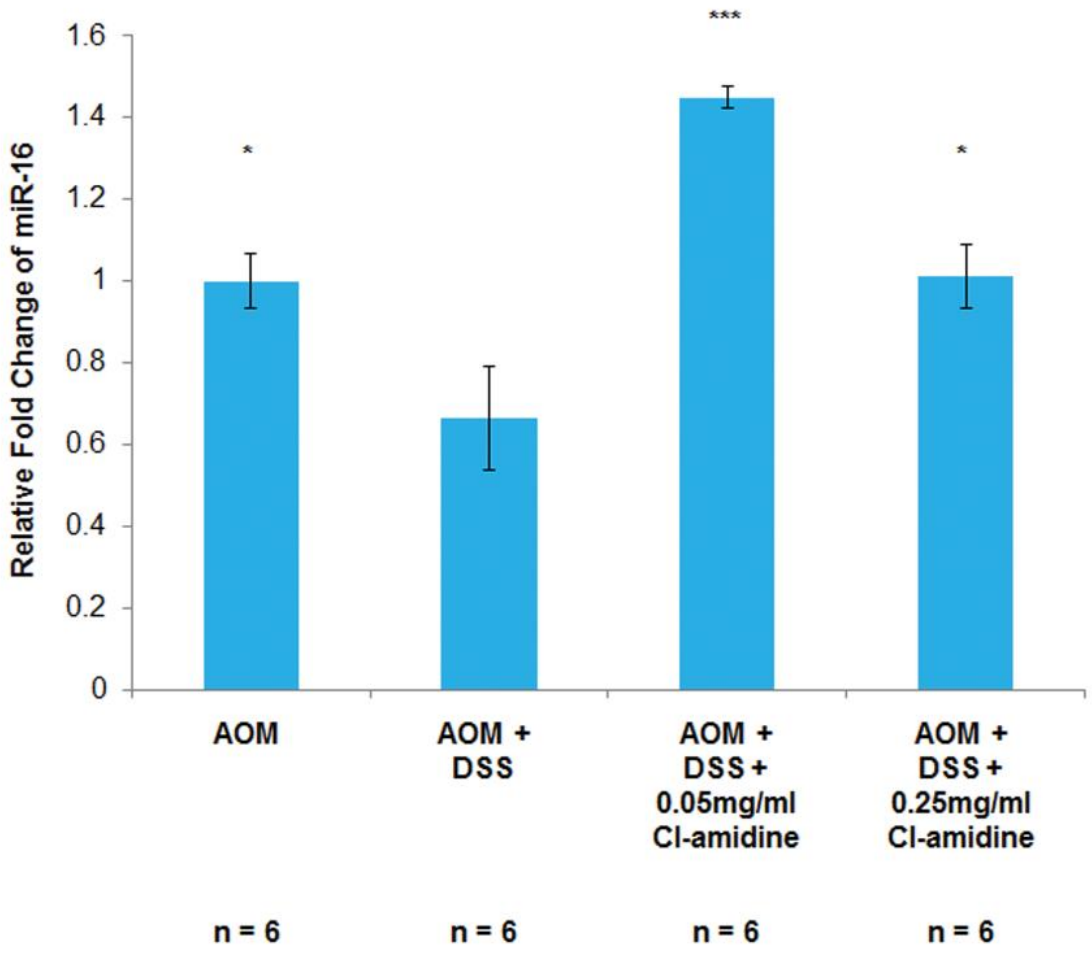

Figure 4.3. MiR-16 expression is increased in the colon epithelial cells of mice treated with Cl-amidine. Mice from each group were euthanized at *day 35* and colons were removed to be processed to separate the colon inflammatory cells from the epithelial cells via magnetic microbeads. Then, as described in the methods, total RNA was extracted and primed to measure miR-16 expression using qPCR. MiR-16 expression across the treatment groups was quantified as the relative fold change as compared to AOM only. MiR-16 expression in colon epithelial (CD45-) cells at *day 35* in the AOM/DSS model of colon cancer is shown. Significant differences from the AOM + DSS only group are indicated by * ($p<0.05$) and *** ($p<0.005$).

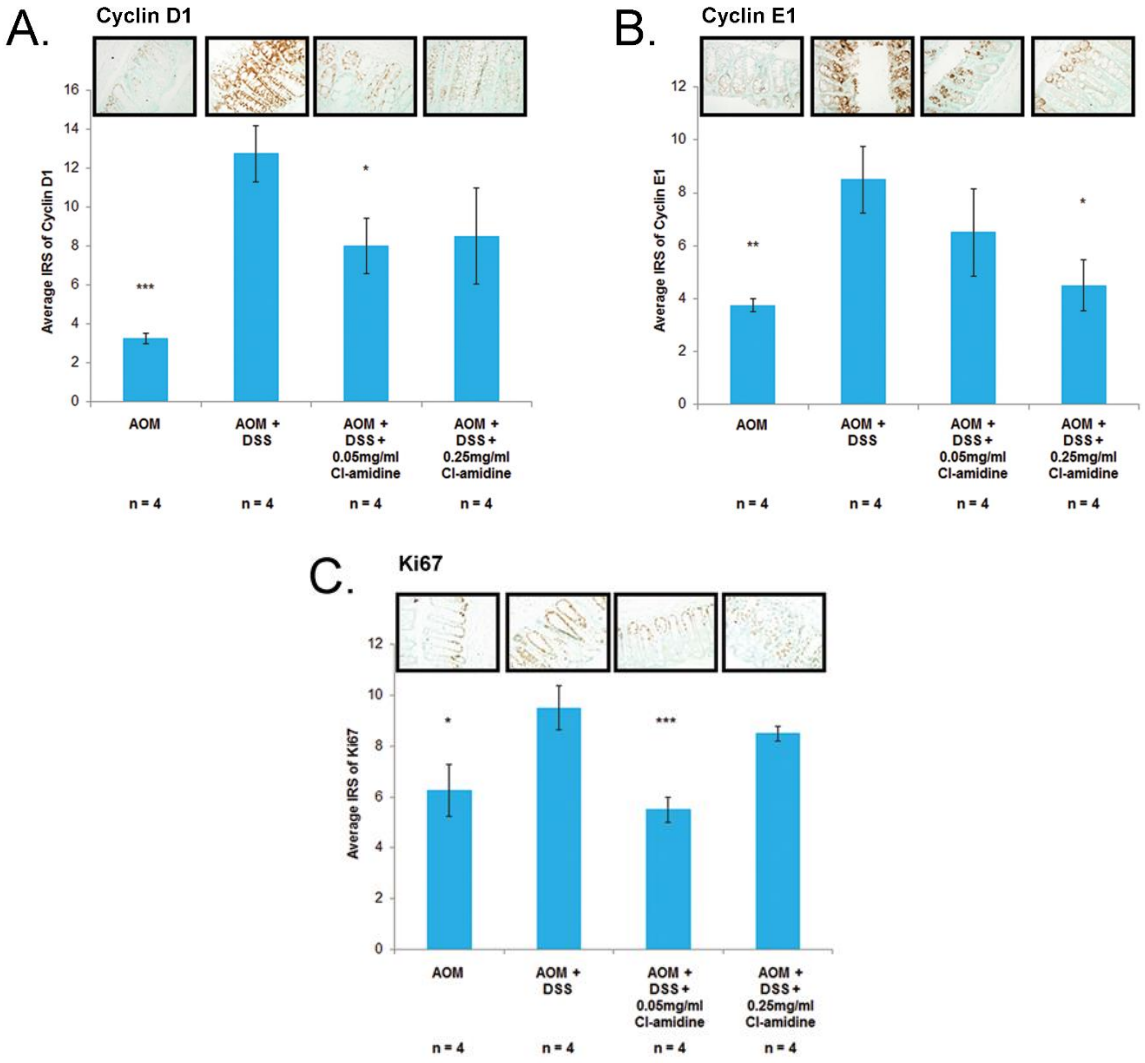

Figure 4.4. Cell proliferation proteins (Cyclin D1, Cyclin E1, and Ki67) are downregulated in mice treated with Cl-amidine. Colons from 4 mice per group were euthanized at *day 35* and processed for IHC analysis. IRS of colons at *day 35* stained with **(A)** Cyclin D1, **(B)** Cyclin E1, both known targets of miR-16, and **(C)** Ki-67, a cell proliferation marker. Representative sections of stained colons (400x total magnification). Significant differences from the AOM + DSS only group are indicated by * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.005$).

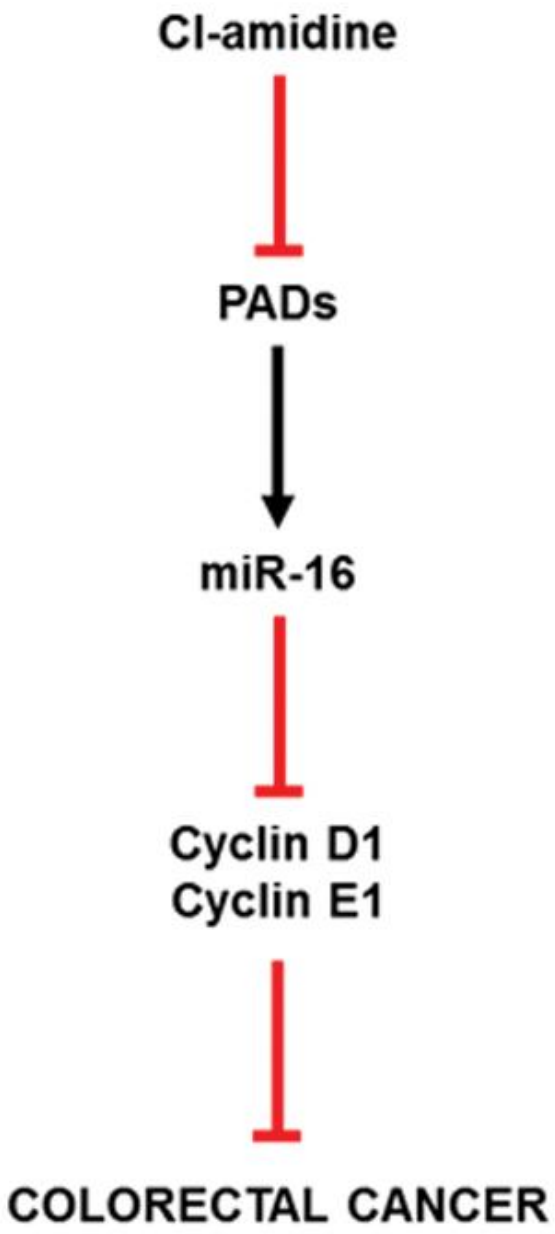

Figure 4.5. Proposed mechanism of action for Cl-amidine preventing CRC. As evidenced in the results produced in this manuscript, Cl-amidine upregulates miR-16 expression in mice through PAD inhibition. At this point, the cell proliferation targets (Cyclin D1 and E1) of miR-16 are downregulated, accounting for the decreased tumorigenesis found at the endpoint of our study.

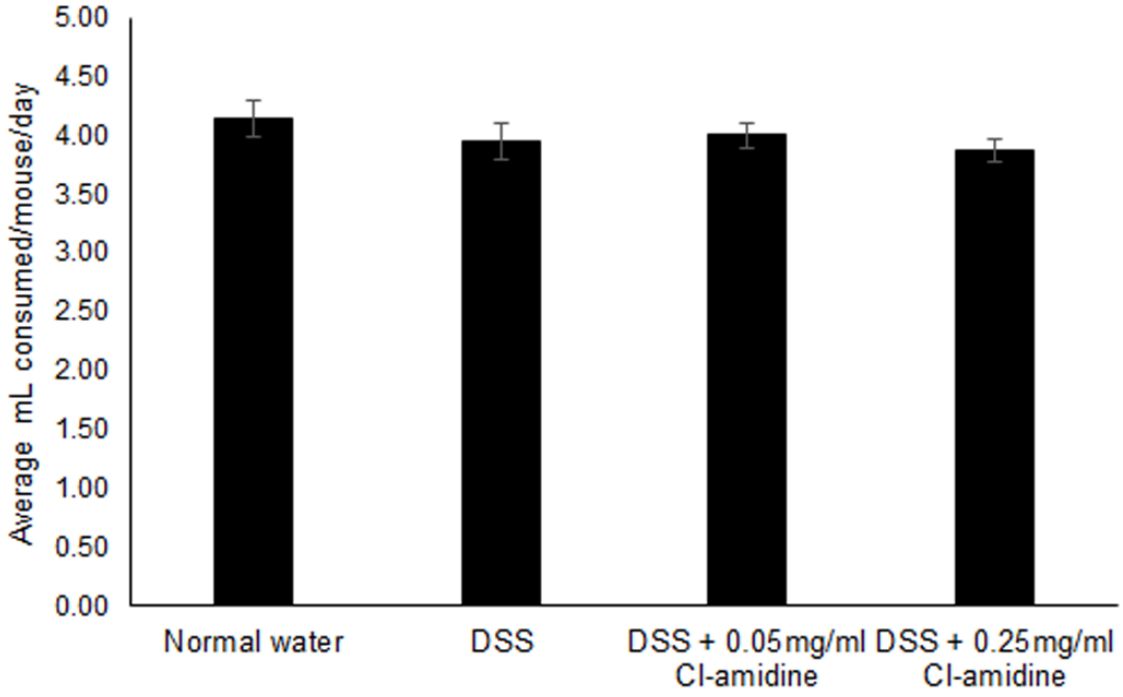

Figure 4.6. Average water intake for mice given 2% DSS and Cl-amidine treatments. Mice were given indicated treatments in water to represent treatments used in the *in vivo* model of colitis-associated colorectal cancer. Daily water consumption was measured for 7 days (Normal water and DSS only groups) and 10 days (DSS + Cl-amidine groups). No significant differences were reported between the groups measured.

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CHAPTER 5

EPIGENETIC REGULATION OF THE TUMOR SUPPRESSOR MIRNA, MIR-16, BY CL-AMIDINE

ABSTRACT: Protein arginine deiminases (PADs) are a family of enzymes that catalyze citrullination, the post-translational conversion of peptidyl-arginine to peptidyl-citrulline. The resulting shift from the neutral arginine to the positive citrulline can interrupt hydrogen bond formation and protein-protein interactions with consequential effects on protein function. PADs and citrullination are associated with the regulation of genes and apoptosis under physiological conditions; however, elevated levels of citrullinated proteins are implicated in the initiation and progression of many inflammatory diseases, including ulcerative colitis and various cancers. Due to the involvement of PADs in many pathological conditions, PAD inhibitors have been developed to prevent and treat abnormal PAD activity. One such small molecule inhibitor of PADs, Cl-amidine, targets the four active isozymes of PADs by covalent modification of the active site of the enzymes. Our previous *in vitro* studies using microarray analysis of miRNAs revealed that only a small subset of miRNAs are affected by Cl-amidine, instead of global miRNA regulation. The most significantly upregulated miRNA by Cl-amidine was miR-16, a putative tumor suppressor miRNA with cell cycle targets. The aim of our current study is to understand how Cl-amidine is targeting select miRNAs, like miR-16. One hypothesis explores the epigenetic regulation of miRNAs, specifically by methylation-mediated gene silencing. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), a family of enzymes

including DNMT1, DNMT3A, and DNMT3B. DNMTs regulate genes by methylating CpG islands located in the promoter regions, while aberrant hypermethylation, possibly caused by citrullination of DNMTs, can trigger methylation-mediated gene silencing of targeted genes and even miRNAs. DNMT hyperactivity and tumor suppressor miRNA downregulation are both associated with various cancers. We propose that abnormal DNMT activity is resulting in the hypermethylation of the miR-16 promoter and is causing its suppression in cancer. Our results indicate that DNMT1 can be citrullinated, perhaps contributing to abnormal DNMT activity. We also found that inhibition of DNMTs was able to rescue miR-16 expression to a comparable level seen upon inhibition of citrullination. Ultimately, Cl-amidine was able to reduce the methylation of the miR-16 promoter as tested with methylation-specific PCR. Altogether, our current study establishes a novel epigenetic facet of Cl-amidine and provides further insight into the complex pathways that are involved in cancer progression.

5.1 INTRODUCTION

Protein arginine deiminases (PADs) are a family of enzymes that catalyze the posttranslational modification, citrullination. Citrullination is the conversion of peptidyl-arginine to peptidyl-citrulline, resulting in a change in charge from the neutral arginine to the positive citrulline residue. Shifts in amino acid charge can interrupt hydrogen bond formation and protein-protein interactions with consequential effects on protein function. PADs and citrullination are associated with regulation of genes, apoptosis, and cellular structure under typical physiological conditions (1-5). In contrast, elevated levels of citrullinated proteins are implicated in the initiation and progression of many inflammatory diseases, including multiple sclerosis (6), rheumatoid arthritis (7, 8), ulcerative colitis (9, 10), and various cancers (11).

Due to the involvement of PADs in many pathological conditions, PAD inhibitors have been developed to prevent and treat abnormal PAD activity. One such small molecule inhibitor of PADs, N-α-benzoyl-N5-(2-chloro1-iminoethyl)-L-ornithine amide (Cl-amidine), targets the four active isozymes of PADs (i.e. PAD1-4) by covalent modification of the active site of the enzymes. Our previous studies have shown that Cl-amidine effectively suppresses inflammation and oxidative stress associated with ulcerative colitis and prevents tumorigenesis of colitis-associated colon cancer (12, 13). Based on our results, we proposed a cancer preventative mechanism of action of Cl-amidine involving the upregulation of the well-studied tumor suppressor microRNA (miRNA), miR-16, and the subsequent downregulation of its cell cycle targets, Cyclin D1 and E1 (13-17). A microarray analysis of miRNAs revealed that only a small subset of miRNAs are affected

by Cl-amidine, instead of global miRNA regulation (14). The aim of our current study is to understand how Cl-amidine is targeting select miRNAs, like miR-16.

Epigenetic regulation of genes has entered the spotlight recently as methylation and other modifications to DNA and histones have become more studied. DNA methylation is catalyzed by a family of enzymes called the DNA methyltransferases (DNMTs). The enzymatically active members of the DNMT family include an abundant maintenance methyltransferase, DNMT1, and *de novo* methyltransferases, DNMT3A and DNMT3B. DNMTs regulate genes by methylating CpG islands located in the promoter regions, while aberrant hypermethylation can trigger methylation-mediated gene silencing of targeted genes and even miRNAs. DNMT hyperactivity and tumor suppressor miRNA downregulation are both associated with various cancers (18-21). For example, the promoter of the tumor suppressor miR-200b can be hypermethylated by DNMT1 and, subsequently, the loss of miR-200b expression results in tumorigenesis (21). We hypothesize that a similar mechanism, initiated by abnormal DNMT activity, is resulting in the hypermethylation of the miR-16 promoter and is causing its suppression in cancer. Abnormal DNMT activity may be due to citrullination since DNMT3A has been reported to be a citrullinated target of PAD4, which promoted elevated levels of methylation (22).

The connection between citrullination and methylation-mediated gene silencing has been considerably under studied, but our data links DNMT citrullination and inhibition to the regulation of miR-16. Our current study demonstrates a novel epigenetic facet of Cl-amidine and provides further insight into the complex pathways that are involved in cancer progression.

5.2 Materials and Methods

5.2.1 Cl-amidine

The synthesis of Cl-amidine has previously been described (23, 24).

5.2.2 Cell Culture and Treatment

The isogenic human colorectal carcinoma cell lines, HCT116 wild-type (wt), DNMT1 -/-, and DNMT3B -/- (see Figure 5.1 for cell line verification), were maintained in McCoy's 5A media (ATCC) supplemented with 10% fetal bovine serum (Life Technologies), penicillin (10 U/mL) and streptomycin (10 µg/mL; Mediatech). To inhibit citrullination, HCT116wt cells were treated with 50 µM Cl-amidine for 12 hours. For experiments involving 5-Azacytidine (5-Aza; Sigma-Aldrich), cells were treated with 10 µM 5-Aza for 24 hours. During the 24 hour treatment, media was changed and replenished with fresh 5-Aza every 12 hours.

5.2.3 Western Blot Analysis and Antibodies

Western blots were performed as previously described (25). The following antibodies were used: α-peptidyl-citrulline (clone F95) (mouse monoclonal, diluted 1:100; EMD Millipore), DNMT1 (rabbit monoclonal, diluted 1:500; Cell Signaling), DNMT3A (rabbit polyclonal, diluted 1:500; Abcam), DNMT3B (mouse monoclonal, diluted 1:500; Novus Biologicals), and GAPDH (clone 14C10) (glyceraldehyde 3-phosphate dehydrogenase; rabbit monoclonal, diluted 1:1000; Cell Signaling).

5.2.4 Immunoprecipitation

Immunoprecipitation was performed according to Dynabeads Protein G (Life Technologies) product description. Briefly, 1.5 mg of magnetic Dynabeads were bound to primary antibodies (DNMT1 diluted 1:50, Novus Biologicals; DNMT3A diluted 1:50,

Abcam; DNMT3B diluted 1:50, Novus Biologicals) for 1 hour at room temperature while rocking. The bead-antibody complex was washed with 1x PBST and then incubated with whole cell protein lysate at room temperature for 1 hour while rocking. Next, the bead-antibody-antigen complex was washed again and resuspended in SDS Loading Buffer (5% β-mercaptoethanol; Life Technologies). To remove the beads from the complex, the samples were boiled for 10 minutes.

5.2.5 Quantitative Real-Time PCR

5.2.5.1 Measuring miRNA Expression

Total miRNA was extracted from HCT116wt, DNMT1 -/-, and DNMT3B -/- cells according to miRNeasy Mini Kit instructions (Qiagen). Quantification of RNA concentration and stability were determined by Nanodrop 2000 (Nanodrop, Wilmington, DE). To reverse transcribe 10 ng of total RNA into cDNA, we used the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to kit instructions. Primers used include hsa-miRNA-16 for miR-16 detection and small nuclear protein RNU6B (U6) for normalization (Applied Biosystems, Foster City, CA). Quantitative Real-Time PCR was performed using the TaqMan miRNA Assay (Applied Biosystems, Foster City, CA) with the 7300 PCR Assay System (Applied Biosystems, Foster City, CA). The relative fold change (FC) of miR-16 expression, as compared to U6 expression, was determined based on the comparative threshold cycles (Ct) of miR-16 and U6. Experiments were performed at three separate times and each sample was analyzed in triplicate.

5.2.5.2 Measuring mRNA Expression

RNA was extracted from HCT116wt cells (untreated or treated with 50 µM Cl-amidine for 12h) according to RNeasy Mini Kit instructions (Qiagen). RNA concentration and stability were measured as described in the previous section. 1 µg of total RNA was used to make cDNA using the Promega Reverse Transcription Kit (Promega Corp, WI) according to kit instructions. The human primer sequences used for Real-Time PCR were: DNMT1 Forward 5'-GAG CTA CCA CGC AGA CAT CA-3', DNMT1 Reverse 5'-CGA GGA AGT AGA AGC GGT TG-3', DNMT3A Forward 5'-GCC TCA ATG TTA CCC TGG AA-3', DNMT3A Reverse 5'-CAG CAG ATG GTG CAG TAG GA-3', DNMT3B Forward 5'-CCC ATT CGA GTC CTG TCA TT-3', DNMT3B Reverse 5'-GGT TCA CAA CAG CAA TGG ACT-3', GAPDH Forward 5'-GAG TCA ACG GAT TTG GTC GT-3', and GAPDH Reverse 5'-GAC AAG CTT CCC GTT CTC AG-3' (Integrated DNA Technologies, Inc.). Quantitative Real-Time PCR was performed using the 7300 Real-Time PCR Assay System (Applied Biosystems, CA) with Power SYBR green PCR master mix (Applied Biosystems, CA) and primers for DNMT1, DNMT3A, DNMT3B, and GAPDH. GAPDH gene expression was used for normalization versus DNMT1, DNMT3A, and DNMT3B. The fold change of DNMT1, DNMT3A, and DNMT3B mRNA expression, as compared to GAPDH mRNA expression, was determined based on the comparative Ct values. Experiments were performed at three separate times and each sample was analyzed in triplicate.

5.2.6 DNA Extraction and Bisulphite Conversion

Genomic DNA (gDNA) was extracted from HCT116wt cells according to Quick-gDNA MiniPrep kit instructions (Zymo Research). To ensure adequate DNA extraction,

DNA concentrations and stability were measured using Nanodrop 2000. Then, 1 µg of gDNA was bisulphite converted using EZ DNA Methylation-Gold Kit (Zymo Research) according to kit instructions.

5.2.7 Methylation-Specific PCR

PCR was performed using eluted bisulphite-converted DNA and methylation-specific PCR primers. The sequences for methylated- and unmethylated-specific primers, as designed with MethPrimer (26), were as follows: Methylated Forward 5'-GGA TAG GTT ATT TTG TTT TTT TCG T-3', Methylated Reverse 5'-CCG ACA TTC CCT AAA ACG AC-3', Unmethylated Forward 5'-GGA TAG GTT ATT TTG TTT TTT TTG T-3', Unmethylated Reverse 5'-TTC CCA ACA TTC CCT AAA ACA-3' (Integrated DNA Technologies, Inc.). The methylation-specific PCR primers were designed to target a miR-16 promoter region near the transcriptional start site of *DLEU2* gene (27). To analyze the methylation status of the targeted miR-16 promoter region, we performed conventional PCR. Briefly, ZymoTaq PreMix (Zymo Research) polymerase mastermix was mixed with corresponding forward and reverse primers (1 µM final concentration), 150 ng of bisulphite-converted DNA template, and nuclease-free water. After conventional PCR, we ran the PCR products on a 1.5% agarose gel with ethidium bromide. Bands were captured under UV light.

5.2.7 Statistical Analysis

Mean differences between groups were compared by Student's T-test. The p-value chosen for significance in this study was 0.05.

5.3 RESULTS

5.3.1 CL-AMIDINE INHIBITS PROTEIN CITRULLINATION AND 5-AZACYTIDINE INHIBITS DNMTS

Protein citrullination has various physiological effects on gene regulation, apoptosis, and structural support; however, under disease conditions, off-target and excessive citrullination can occur (1, 5, 10, 28-30). Aberrant citrullination can result in loss of function, reduced function, or even hyperactivity of a protein. We have shown that Cl-amidine, a pan-PAD inhibitor, decreases peptidyl-citrulline levels in a dose-dependent manner (Figure 5.1C). Our previous studies have also shown that Cl-amidine, at a dose comparable to 50 μM, increases miR-16 expression in HCT116wt cells (14). Interestingly, Cl-amidine was found to alter the expression of only 18 miRNAs in a microarray analysis (14), suggesting that Cl-amidine is not targeting global miRNA expression.

In order to understand how Cl-amidine is selectively affecting certain miRNAs, we investigated a potential epigenetic mechanism of action involving DNMTs. DNMTs (i.e. DNMT1, DNMT3A, and DNMT3B) are key regulators of gene expression and can cause methylation-mediated gene silencing in certain instances of hypermethylated gene promoters (31). To decipher the effects of DNMT activity in our study, we used 5-Aza, a DNMT inhibitor. We saw optimal DNMT inhibition at a concentration of 10 μM for 24h (Figure 5.1D).

5.3.2 DNMT1 IS CITRULLINATED

To verify that DNMTs can be post-translationally citrullinated, we performed immunoprecipitation to isolate individual DNMTs and probed for peptidyl-citrulline. The presence of peptidyl-citrulline in untreated HCT116wt cells indicates that isolated DNMT1

(Figure 5.2A) is citrullinated. Interestingly, DNMT3A and DNMT3B do not appear to be citrullinated (Figure 5.2B and C). Although research concentrating on the citrullination of DNMTs is limited, our findings contradict the article by Deplus *et al*. which found DNMT3A to be citrullinated by PAD4 (22). However, it is important to note that different cell lines were used and DNMT1 and DNMT3B were not examined in previous experiments (22).

5.3.3 Inhibition of DNMTs rescues suppression of miR-16

Since the citrullination of DNMT1 has been validated (Figure 5.2A), we observed the effects of Cl-amidine and 5-Aza treatments on miR-16 levels. Our group has previously shown that Cl-amidine significantly upregulated miR-16 expression *in vitro* and *in vivo* (13, 14). Citrullination can alter protein activity (22, 32, 33) and Deplus *et al.* specifically showed that the citrullination of DNMT3A resulted in hypermethylation (22). Furthermore, hypermethylation of gene promoters can lead to methylation-mediated gene silencing (31). To determine if Cl-amidine is working by preventing methylation-mediated gene silencing of miR-16, we must first consider if the inhibition of DNMTs, by 5-Aza treatment, is sufficient to increase miR-16 expression. In fact, our results reveal that 5-Aza treatment significantly increased miR-16 expression to a level comparable to Cl-amidine treatment (Figure 5.3A), possibly by similar regulation of DNMTs.

To further delineate which of the DNMTs is/are responsible for miR-16 suppression, we measured miR-16 expression in HCT116wt versus HCT116 DNMT1-/- and HCT116 DNMT3B-/- cell lines. miR-16 expression was significantly upregulated, as compared to levels in HCT116wt, only in HCT116 DNMT1-/- cells (Figure 5.3B). This finding indicates that DNMT1 is the DNMT essential for modulating miR-16 expression.

Furthermore, DNMT1 gene expression is significantly more abundant than DNMT3A and DNMT3B (Figure 5.3C), making its regulation of greater interest and consequence.

5.3.4 Methylation of the miR-16 promoter

miR-16 is a putative tumor suppressor miRNA with various cell cycle targets (14-17, 34) and is frequently deleted or suppressed in various cancers. For instance, in chronic lymphocytic leukemia (CLL), miR-16 and its host gene, *DLEU2*, are frequently deleted (35); whereas, in breast and colon cancer, miR-16 is often downregulated (36-38). Based on our results thus far, our hypothesis is that miR-16 is being epigenetically downregulated in colon cancer by the hypermethylation of its promoter. To test this theory, we extracted gDNA from HCT116wt samples for bisulphite conversion. Then, using methylated and unmethylated primers, we performed methylation-specific PCR targeting the promoter sequence of miR-16 on its host gene, *DLEU2* (Figure 5.4A). We found that Cl-amidine (50 μM for 12h) and 5-Aza (10 μM for 24h) decreased the expression of the methylated miR-16 promoter as compared to the untreated HCT116wt cells (Figure 5.4B). In accordance with our hypothesis, we see that Cl-amidine reduces methylation of the miR-16 promoter, indicating that Cl-amidine works to rescue miR-16 expression by alleviating the methylation-mediated silencing caused by hyperactive DNMTs.

5.4 Discussion

As we show in our current study, citrullination occurs in DNMT1, but not DNMT3A and DNMT3B (Figure 5.2). The selectivity towards citrullinated DNMT1 could be due to the shear abundance of DNMT1 as compared to DNMT3A and DNMT3B in the cell or more potential sites of citrullination (i.e. more arginine residues present). We also see that DNMT inhibition causes an increase in the tumor suppressor miR-16, similar to

the rescue of miR-16 seen in Cl-amidine-treated cells (Figure 5.3A). Thus, our results indicate that Cl-amidine may indeed be acting to regulate DNMTs as well. We found that DNMT1 inhibition alone was able to restore miR-16 expression (Figure 5.3B). Since DNMT1 has higher basal expression than DNMT3A and DNMT3B (Figure 5.3C), the importance of its hypothesized role in the epigenetic regulation of miR-16 is amplified. Finally, we establish that the methylation status of the miR-16 promoter is inversely related to miR-16 expression, as Cl-amidine and 5-Aza caused reduced promoter methylation (Figure 5.4B) and increased miR-16 expression (Figure 5.3A).

Epigenetic regulation of genes has proven to have a substantial effect on tumor initiation, progression, and metastasis. A major focus of epigenetic studies is the modification of DNA and histones. Methylation is a well-studied epigenetic factor that can alter gene expression with as little as the addition or removal of methyl groups on a histone or DNA (31, 39). Another factor that is emerging as an epigenetic modulator is citrullination. Citrullination has been reported by many researchers to target histones (40-44), but more recently, only a single study has been published to indicate DNMTs (specifically DNMT3A) can also be targeted by PADs (22). Based on our previously published data implicating that Cl-amidine can increase expression of several tumor suppressor miRNAs and can prevent UC-associated CRC (13, 14), we decided to further explore the effects of DNMT citrullination in our human colorectal cell line. To date, our group is the first to indicate that Cl-amidine may be able to rescue miRNA expression through the regulation of DNMT activity. Since the majority of genes are targets of miRNAs (45), our work provides a novel pathway connecting PAD inhibition with downstream DNMT regulation to alleviate methylation-mediation gene silencing.

Collectively, our findings support the hypothesis that Cl-amidine upregulates miR-16 expression by inhibiting the citrullination and hyperactivity of DNMTs, which in turn eliminates methylation-mediated silencing of miR-16 (Figure 5.5). Results from this study reveal the effects of Cl-amidine on the promoter methylation activity of DNMTs. Overall, the potential for Cl-amidine as a drug to prevent colitis-associated colorectal cancer is strengthened by our study which reveals an innovative mechanism of epigenetic regulation by Cl-amidine.

5.5 Future Experiments and Rationale

In our current study, we reported that DNMT1 is key to inhibiting miR-16 expression (Figure 5.3B). However, we do not have a cell line that is DNMT3A deficient and, therefore, cannot rule out that DNMT3A may also be essential to regulating methylation-mediated silencing. To solve this problem, we could use siRNA to individually knockdown DNMT1, DNMT3A, and DNMT3B. Using siRNA would allow us to investigate the individual roles that all 3 major DNMTs (DNMT1, DNMT3A, and DNMT3B) have on miR-16 expression. Moreover, to further substantiate our current results showing that Cl-amidine is reducing DNMT hyperactivity [i.e. miR-16 downregulation (Figure 5.3A) and hypermethylation of miR-16 promoter (Figure 5.4B)], we propose to treat siRNA knock down cells with Cl-amidine and then measure total methylation using an ELISA-based methylation assay kit (Epigentek). This assay kit uses a colorimetric reaction to quantify total 5-methylcytosine (5-mC) content in gDNA samples. Results from this experiment will determine if Cl-amidine significantly inhibits hypermethylation through the regulation of one or more DNMTs.

This study could also be extended to other miRNAs that we have shown to be upregulated by Cl-amidine *in vitro* (e.g. miR-200b, let-7a, miR-19b) (14). To look at these other miRNAs, we would need to design unmethylated- and methylated-specific primers for a promoter sequence for each of these miRNAs in a similar fashion to how the miR-16 methylation-specific primers were designed (Figure 5.4). Then, using bisulphite-converted gDNA, we could compare the promoter methylation of each of these miRNAs for untreated, Cl-amidine-treated, and 5-Aza-treated samples [same method as we completed for miR-16 methylation-specific PCR (Figure 5.4B)]. If Cl-amidine is increasing miRNA levels by inhibiting promoter hypermethylation, then we would expect to see the methylation of these miRNA promoters decreased too. Furthermore, to validate that Cl-amidine is not inhibiting promoter methylation in all miRNAs, we could use methylation-specific primers for miRNAs whose expression was not significantly altered by Cl-amidine (14). For example, we could use tumor suppressor miRNAs (i.e. miR-29b, miR-143, and miR-145) that have been shown to be downregulated in colon cancer (46-49), but were not found to be regulated by Cl-amidine treatment in our microarray (14). Results from this experiment would also provide evidence that Cl-amidine is not affecting global miRNA expression. Ultimately, these additional experiments are proposed to strengthen our current *in vitro* study and to lay the ground work for future *in vivo* studies exploring the epigenetic regulation by Cl-amidine in animal models.

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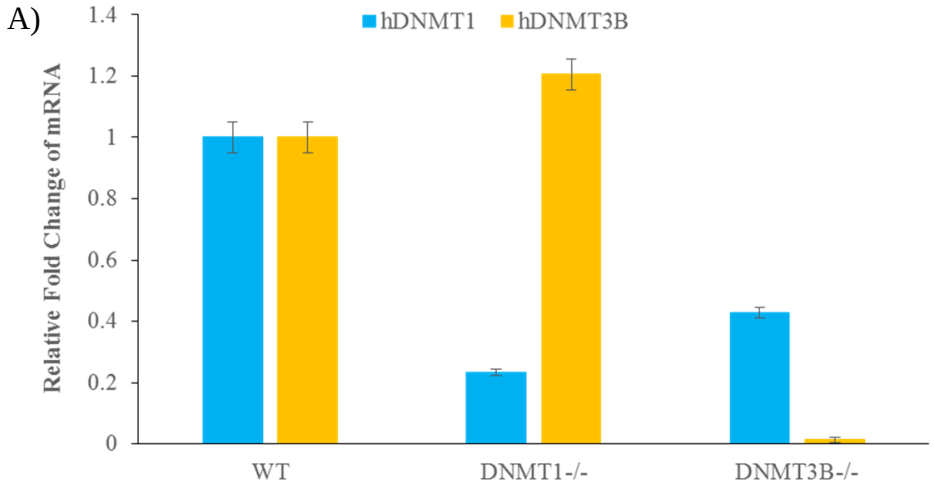
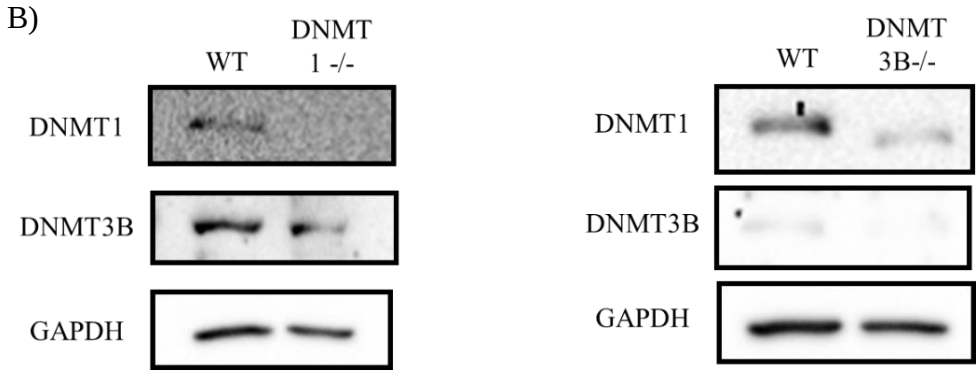
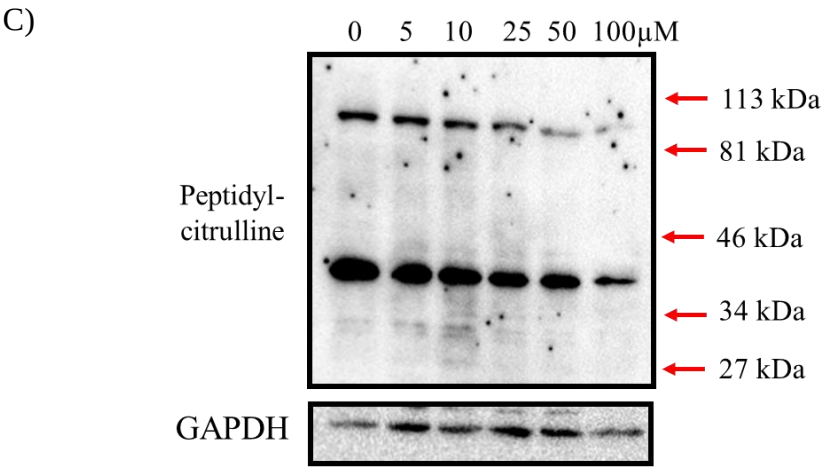
FIGURES


A)
hDNMT1
hDNMT3B
Relative Fold Change of mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
DNMT1-/-
DNMT3B-/-


B)
WT
DNMT 1 -/-
DNMT1
DNMT3B
GAPDH
WT
DNMT 3B-/-
DNMT1
DNMT3B
GAPDH


C)
0 5 10 25 50 100µM
Peptidyl-citrulline
113 kDa
81 kDa
46 kDa
34 kDa
27 kDa
GAPDH

D)

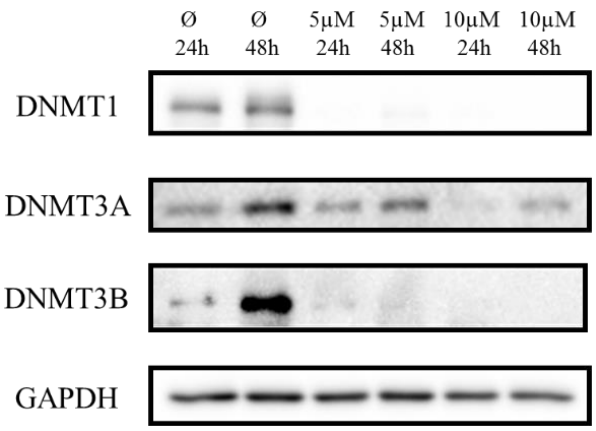
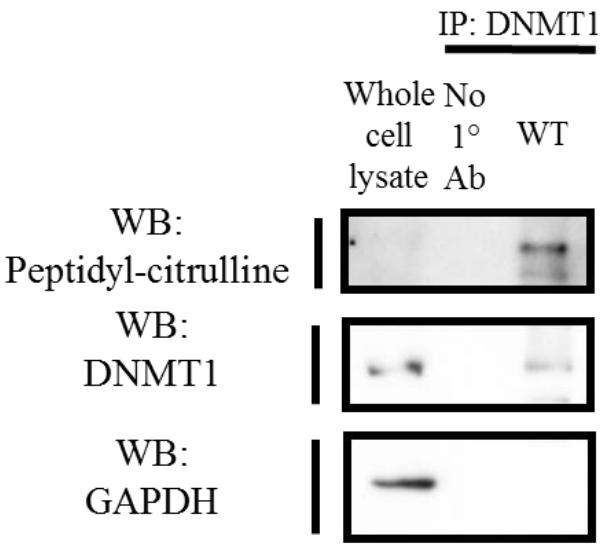
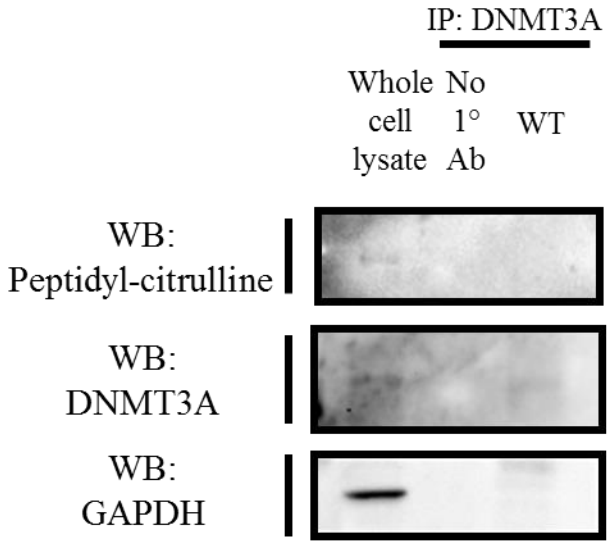


Figure 5.1. Cell line and treatment verification. DNMT mRNA **(A)** and protein **(B)** expression in HCT116 DNMT1-/- and DNMT3B-/- cell lines as measured by qPCR and Western Blot analysis, respectively. **C)** Cl-amidine inhibits peptidyl-citrulline expression in a dose-dependent manner and optimally for our experiments at 50 μM for 12h in HCT116wt. **D)** 5-Aza inhibits DNMT1, DNMT3A, and DNMT3B expression at 10 μM for 24h in HCT116wt.

A)


IP: DNMT1
Whole cell lysate
No 1° Ab
WT
WB: Peptidyl-citrulline
WB: DNMT1
WB: GAPDH

B)


IP: DNMT3A
Whole cell lysate
No 1° Ab
WT
WB: Peptidyl-citrulline
WB: DNMT3A
WB: GAPDH

C)

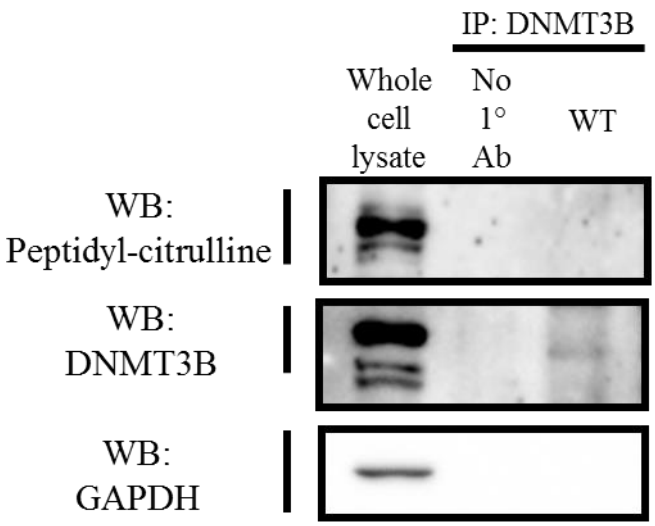
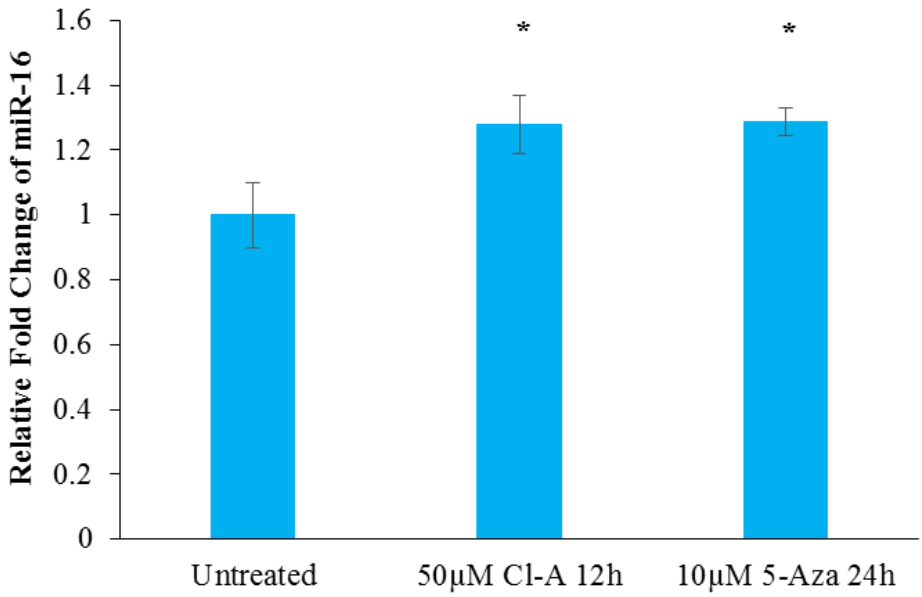
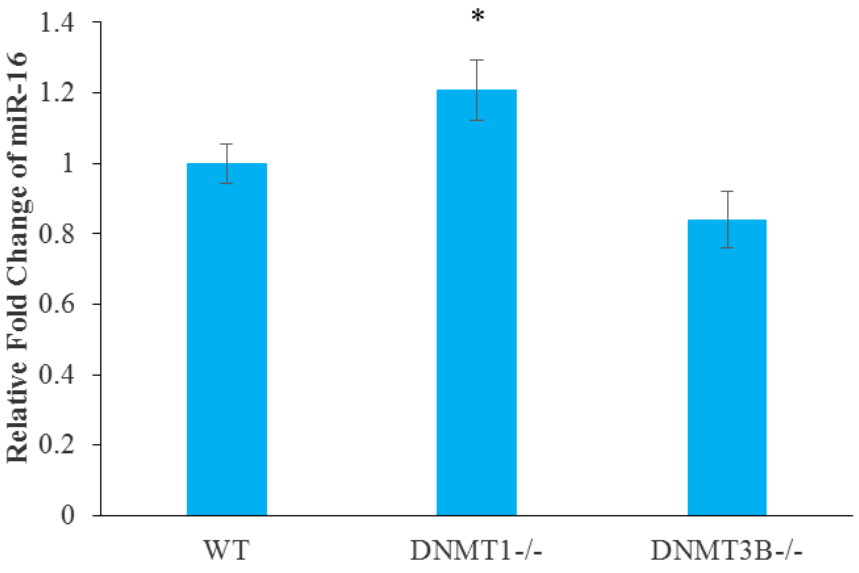


Figure 5.2. The citrullination of DNMTs. DNMT1 **(A)**, but not DNMT3A **(B)** and DNMT3B **(C)**, is shown to be citrullinated. Citrullination of immunoprecipitated DNMTs was measured by probing with peptidyl-citrulline antibody. The immunoprecipitation control (No 1° Ab) indicates samples that were incubated with IP beads without bound primary antibody.

A)


Relative Fold Change of miR-16
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Untreated
50μM Cl-A 12h
10μM 5-Aza 24h

B)


Relative Fold Change of miR-16
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
DNMT1-/-
DNMT3B-/-

C)

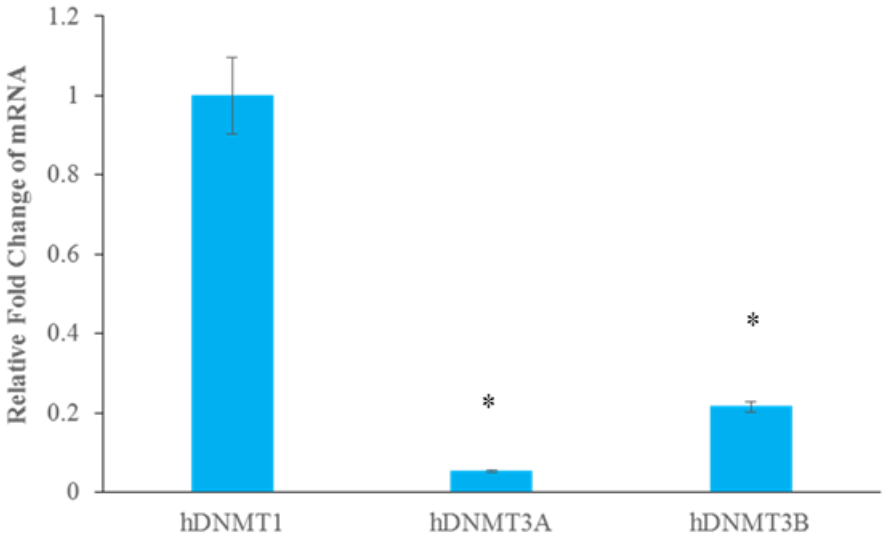
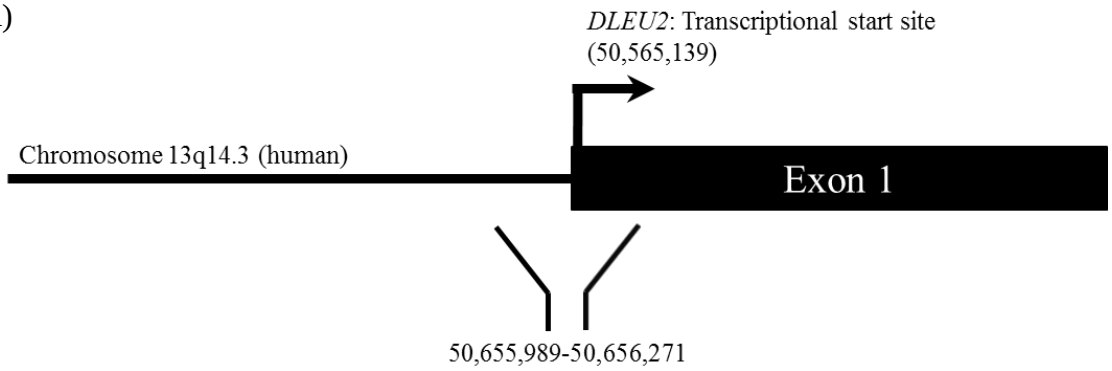


Figure 5.3. The effects of DNMT inhibition and knockout on miR-16 expression. A) 5-Azacytidine treatment (10 µM for 24h) increases miR-16 expression to levels comparable to after Cl-amidine treatment (50 µM for 12h) in HCT116wt cells. **B)** miR-16 expression is increased in HCT116 DNMT1-/-, but not HCT116 DNMT3B-/- cells, as compared to HCT116wt cells. **C)** DNMT1 gene expression is substantially more abundant than DNMT3A and DNMT3B in HCT116wt cells. Significant differences from the Untreated (A), WT (B), or hDNMT1 (C) samples are indicated by * ($p<0.05$).

A)



GACTGCCCTTTGCTCCAAGTAATTTTTGGCGATTTTTAAAGTAATTTT
TCCGGCGGAGTCATAGTGGCGCTATACTCTCGGACAGGTTATCCTGT
CTCTCCCGCTAGATTGATGAGATCAGGCATCACTCGAAAATGGCGCC
GAAAGCGCTGAGGCTCCTCATTCAAATTCGTTAGAGCCAACCCCGCC
GCCCCAGGGAATGCCGGGAACGCGGGCTGCCTCCACAGCTGTCAAT
ACCGCCTCTTAACCCCCCTTCTCTTGCTTTCCCGACATTTTTACGCAT

B)

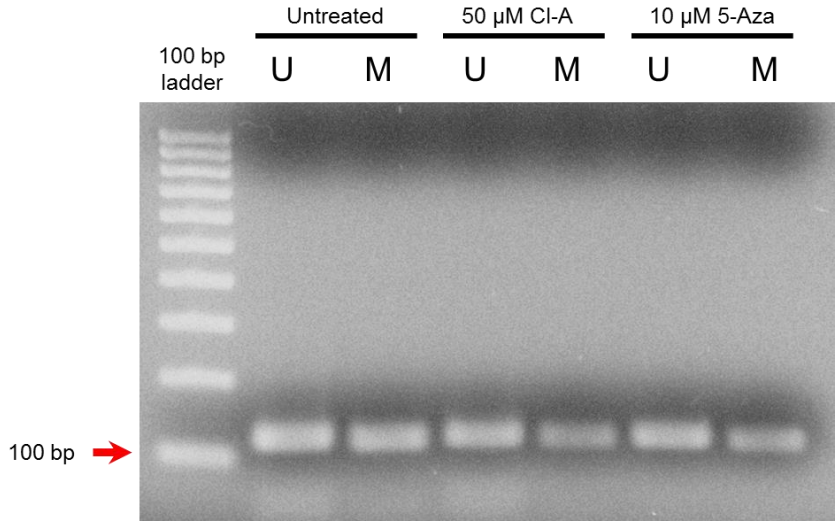


Figure 5.4. The effect of Cl-amidine on the methylation status of the miR-16 promoter. A) The nucleotide sequence (50,655,989-50,656,271) is a truncated, yet active, *DLEU2* promoter sequence (27). The promoter sequence also occurs within a CpG island (50,655,070-50,656,564); therefore, this sequence can be used to build methylation-specific PCR primers for the promoter of *DLEU2*/miR-16. Using the MethPrimer online tool and database (26), this promoter sequence can be entered to get sets of Unmethylated- and Methylated-specific PCR primers. The nucleotide at the transcriptional start site of *DLEU2* is highlighted in green. **B)** Sets of unmethylated (U; 130bp) and methylated (M; 127bp) primers were used to observe the methylation status of the miR-16 promoter in untreated HCT116wt cells versus cells treated with 50 µM Cl-amidine for 12h or 10 µM 5-Aza for 24h. Based on the intensity of the bands, samples treated with Cl-amidine and 5-Aza both seem to reduce the expression of the methylated miR-16 promoter as compared to the untreated samples. The DNA ladder indicates 100bp at each band.

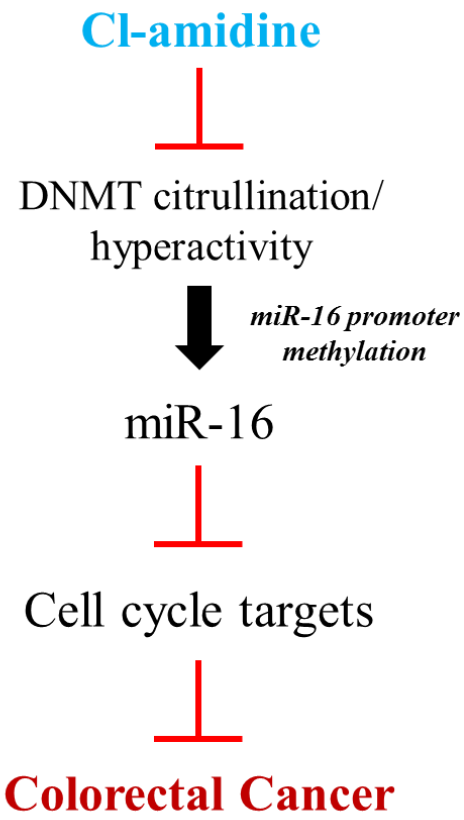


Figure 5.5. Proposed model of the epigenetic regulation of miR-16 by Cl-amidine. Our results demonstrate that the inhibition of DNMT citrullination/subsequent hyperactivity can rescue miR-16 expression in HCT116 colon cancer cells. The resulting increase in miR-16 expression is due to the reduced methylation levels found at the miR-16 promoter after Cl-amidine treatment. The restored expression of once silenced (by methylation) tumor suppressor miRNAs will downregulate oncogenic targets and will ultimately prevent the initiation of carcinogenesis.

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CHAPTER 6

MECHANISM OF ACTION FOR INHIBITION OF PAD-RELATED COLORECTAL CANCER: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 SUMMARY AND CONCLUSIONS

In Chapter 3, we showed that Cl-amidine was able to reduce inflammatory markers, iNOS and Cox-2, and oxidative stress in the ANA-1 murine macrophage cell line. Meanwhile, in a pro-inflammatory environment, DNA damage was inhibited by Cl-amidine in the HCT116wt colon cancer cell line. We then turned to an *in vivo* model of colitis. In the AOM/DSS mouse model of ulcerative colitis (UC), we were able to corroborate similar results from our *in vitro* experiments, demonstrating that our cellular models of inflammation are adequate for initial studies and are translatable to our animal model (1).

Building off of our success in Chapter 3, we followed the pathological progression from UC to colorectal (CRC) and thus expanded our research focus to include studying Cl-amidine as a prospective cancer preventative. Initial *in vitro* results brought the importance of tumor suppressor miRNAs, mainly miR-16, into the picture. In Chapter 4, we reported that miR-16 was upregulated by Cl-amidine and caused subsequent G1 cell cycle arrest *in vitro*. We also performed PAD4 and miR-16 knock downs to verify that the outcomes seen with Cl-amidine treatment are indeed occurring as a direct result of PAD4 inhibition and that G1 cycle arrest is being mediated by miR-16. These findings set the foundation for our *in vivo* model of UC-associated CRC. Since miR-16 expression was correspondingly upregulated by Cl-amidine *in vitro* and *in vivo*, we were once again able to validate our

cellular results in our animal model. Results from Chapter 4 establish that Cl-amidine is not only a prospective colitis treatment, but it also is favorable as a UC-associated CRC preventative as seen in the suppressed tumorigenesis and upregulation of tumor suppressor miR-16 (2).

We took an epigenetic approach in Chapter 5 to answer the lingering question of why Cl-amidine is only influencing a small subset of miRNAs instead of global miRNA expression. Based on previous reports, we developed the hypothesis that DNA methyltransferase (DNMT) dysregulation is responsible for methylated-mediated silencing of targeted genes. In Chapter 5, we determined that DNMT inhibition and Cl-amidine are able to equally rescue miR-16 expression *in vitro*. Additionally, we showed that miR-16 expression can be increased by DNMT1 inhibition alone, making DNMT1 expression and activity of the greatest significance in our current epigenetic study. Cl-amidine also was capable of reducing the methylation of the miR-16 promoter by preventing dysregulated DNMT activity. Based on our results, we explain that the selectivity of Cl-amidine to regulate only certain miRNAs is based off of each miRNA promoter's susceptibility to hypermethylation and silencing. The results from Chapter 5 implicate PADs/citrullination as a modulator of DNMT activity with subsequent silencing of genes involved in an array of pathways; hence, explaining how Cl-amidine can regulate a multitude of cellular responses to suppress pro-inflammatory pathogenesis and tumorigenesis.

With the findings presented in this dissertation, we have shown that Cl-amidine is in fact an effective anti-inflammatory compound with anti-oxidant properties. Cl-amidine likewise protects epithelial cells from DNA damage and successfully prevents the

progression from UC to CRC. We see that Cl-amidine influences many different processes, which reflects the myriad of pathways also modulated by PAD activity.

6.2 Future Directions

We have successfully uncovered the role of Cl-amidine in numerous pathways, including regulatory effects on ROS production, anti-oxidant enzyme expression, miRNA expression, cell proliferation, and epigenetics through the use of various *in vitro* and *in vivo* models. Due to its minimal toxicity and anti-inflammatory, anti-oxidant, and anti-cancer properties, Cl-amidine has a very promising potential as a therapeutic and preventative against UC and CRC. Future studies can continue testing the efficacy of Cl-amidine in other species, as well as investigating the roles of p53- and miR-16-dependence in animal models. Our group plans to utilize mouse models with genes of interest knocked out. We propose using miR-16 knockout (KO) mice to reveal the importance of miR-16 in the effectiveness of Cl-amidine to prevent UC-associated CRC. miR-16 KO mice have been generated by other labs and are commercially available (3); however, these mice have not been subjects of colitis-induced colorectal cancer models, making our future work groundbreaking.

Additionally, we can knock out PADs, specifically PAD4, to show that Cl-amidine is working directly through PAD inhibition and that PADs are important for the pathogenesis of UC. Currently PAD4 KO mice have been established, but have been used to study rheumatoid arthritis, hematopoietic cells, and non-small cell lung cancer (4-6). Using the PAD4 KO mice to study ulcerative colitis and the progression to colorectal cancer would be a novel and innovative contribution to broaden the understanding of the role of PADs in this inflammatory disease.

Furthermore, future studies will be conducted using the more potent and bioavailable second generation compound, BB-Cl-amidine. BB-Cl-amidine has been shown to be about 20 times more potent than Cl-amidine with a lower EC_{50} (7), but still acts through PAD inhibition to yield similar regulation of pathways also targeted by Cl-amidine. Our current results and future projects will promote the overall understanding of Cl-amidine, and related compounds, and are ultimately aimed at the development of these compounds to help millions of people around the world who are affected by UC and UC-associated CRC.

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