Title Page

Inhibiting protein arginine deiminases has anti-oxidant consequences.

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Running Title Page

a) Running title: Anti-oxidant/anti-DNA damage consequences of PAD inhibition

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c) Number of text pages: 13

Number of tables: 0

Number of figures: 6

Number of references: 43

Word count:
   Abstract: 193
   Introduction: 494
   Discussion: 575

d) Abbreviations:
   AOM (azoxymethane); Cox-2 (cyclooxygenase-2); DSS (dextran sulfate sodium); FBS (fetal bovine serum); GPx1 (glutathione peroxidase 1); IBD (inflammatory bowel disease); IFN-γ (interferon-gamma); iNOS (inducible nitric oxide synthase); PAD (protein arginine deiminase); ROS (reactive oxygen species); SOD1 (superoxide dismutase 1)

e) Recommended section assignment: Gastrointestinal, Hepatic, Pulmonary, and Renal
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.
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 (Itzkowitz and Yio, 2004). 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) (Jones et al., 2009), a process called ‘citrullination’. Mammals encode 5 isozymes within a single evolutionarily conserved gene cluster located on human chromosome 1 (1p35-36) (Vossenaar et al., 2003). 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 (Vossenaar et al., 2003). 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 (Mohanan et al., 2012). 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 (Struyf et al., 2009). We have confirmed that PADs are also overexpressed in the colons of colitis patients and in mouse colitis (Chumanevich et al., 2011). PAD levels have also been reported to be increased in tumors, including colon adenocarcinomas (Chang and Han, 2006; Mohanan et al., 2012).

Importantly, we showed that Cl-amidine (Figure 1), a novel, small molecule PAD inhibitor, delivered by oral gavage, suppresses mouse colitis (Chumanevich et al., 2011). It should be noted that Cl-amidine irreversibly inhibits PADs through the covalent modification of Cys645 in the active site of the enzymes (Knuckley et al., 2010); and due to steric hindrance, can only inhibit activated PADs that have undergone the calcium-dependent conformational change at the active site (Luo et al., 2006).

Because citrullination is inflammation-dependent (Makrygiannakis et al., 2006), PADs cause citrullination, PAD inhibition by Cl-amidine suppresses inflammation, and inflammation is characterized by infiltrating leukocytes releasing free radicals (‘oxidative stress’) (Kim et al., 2012); 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.
Methods

Cl-amidine

The synthesis of Cl-amidine has been described previously (Luo et al., 2006; Causey and Thompson, 2008).

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).

Co-culture conditions

Co-culture experiments were carried out as previously described (Hofseth et al., 2003) 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.

**DSS mouse model of colitis**

An outline of the colitis mouse model can be found in Figure 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 (Reagan-Shaw et al., 2007), 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 (Reagan-Shaw et al., 2007), the human equivalent dose (HED, mg/kg) = animal dose (mg/kg) x \[\text{Animal } \text{Km}/\text{Human } \text{Km}\]. 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.

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 (Jin et al., 2010).

Western blot analysis and antibodies

Western blots were carried out as described previously (Ying et al., 2005). 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).

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 (Poudyal et al., 2012).

**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 (Jin et al., 2008).

**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.

**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.
Results

*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 (Chumanevich et al., 2011). Because Cl-amidine has a relatively short half-life in mice (37 min) (Bicker et al., 2012), we wanted to determine whether adding Cl-amidine in the drinking water would enable a slower delivery of similar dosages that had worked previously (Chumanevich et al., 2011). 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 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 (Bicker et al., 2012).

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 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.

*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 (Luhrs et al., 2002; Sartor, 2006). Since we found that pre-incubating ANA-1 mouse macrophages with Cl-amidine suppressed iNOS and Cox-2 induction by IFN-γ (Figure 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 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 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 (Cetinkaya et al., 2006; Sakthivel and Guruyayoorappan, 2013; Ren et al., 2014). Figure 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 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 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 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 6B). CD45- colon epithelial cells from the same mice were examined for DNA damage by Comet analysis. Figure 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 (Petzold and Swenberg, 1978; Hong et al., 2001). These results indicate that Cl-amidine attenuates inflammatory cell activation and protects from colon epithelial cell damage \textit{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.
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 (Bicker et al., 2012), suppresses colitis (Figure 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 4) and in vivo (Figure 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 (Knuckley et al., 2007; Darrah et al., 2012). 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 (Li et al., 2008; Tanikawa et al., 2009; Guo and Fast, 2011; Yao et al., 2008; Zhang et al., 2011). Because Cl-amidine is a pan-PAD inhibitor (Knuckley et al., 2010), it is likely that it suppresses the citrullination of many of these PAD target proteins.

Since citrullination is an inflammation-dependent event (Makrygiannakis et al., 2006), and we have previously shown (and show here) that Cl-amidine suppresses colon inflammation (Figure 2) (Chumanevich et al., 2011), 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 (Moelants et al., 2013)], but also downstream targets, such as iNOS and Cox-2; both of which
were suppressed by Cl-amidine (Figures 3, 4 and 6). Our finding that catalase, GPx1 and SOD1 are all elevated by Cl-amidine \textit{in vitro} and \textit{in vivo} (Figure 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 (Li et al., 2008; Cui et al., 2013), and p53 drives the expression of anti-oxidant enzymes (Kang et al., 2012; Hussain et al., 2004; Popowich et al., 2010; Tan et al., 1999; Yoon et al., 2004; Bensaad et al., 2006), 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 (Fuss et al., 1999). 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 \textit{in vitro} and \textit{in vivo}.
Acknowledgements
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
References


Chumanevich AA, Causey CP, Knuckley BA, Jones JE, Poudyal D, Chumanevich AP, Davis T, Matesic LE, Thompson PR, and Hofseth LJ (2011) Suppression of colitis in mice by Cl-


Footnotes

This work was supported by the National Institutes of Health [Grant 5R01CA151304].
Legends for Figures

Figure 1. Structure of the pan-PAD inhibitor, Cl-amidine.

Figure 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. 6 mice from each group described in Methods were euthanized on day 14, and colons were measured, harvested. Then the histology score was determined. Weight was recorded every 48h during the 14 day experiment. Values represent the mean ± standard error (SE) of the mean. Representative H&E stained colons are shown for each group. *, indicates a significant difference from the AOM+DSS group (p < 0.01).

Figure 3. iNOS levels are reduced in the colons of mice treated with Cl-amidine. 6 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 methods. (A) Immunoreactivity score (IRS) for each group. Values represent the mean ± SE. *, indicates a significant difference from the AOM+DSS only group (p < 0.01). (B) Representative sections of indicated group. Positive staining is brown colored. 400 x magnification.

Figure 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 following 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 h, 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 h, +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 pre-treatment with Cl-amidine (10 µg/mL). Chemiluminescence was measured as described in methods. Results were compared to no Cl-amidine control (± SE). (C) In the presence of an oxidative burst, target epithelial cells (HCT116 colon cancer cells) pre-treated with 10 µg/mL Cl-amidine are protected from DNA damage. Results are represented as the mean Comet tail moment ± SE, scoring 50 comets/treatment group. Representative images of Comets in each treatment group are shown above each bar graph.

Figure 5. Cl-amidine induces anti-oxidant enzymes (catalase, GPx1, SOD1) in IFN-γ-stimulated ANA-1 mouse macrophages and in vivo. (A) ANA-1 mouse macrophage cells were pre-treated with 0-10 µg/mL Cl-amidine for 12 h, then cells were stimulated with IFN-γ for 8 h. Anti-oxidant enzymes of interest were suppressed in activated cells pre-treated 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 (B) catalase, (C) GPx1, and (D) SOD1 as described in methods. Immunoreactivity scores (IRS) are shown for each group. Values represent the mean ± SE. Significance is compared to the AOM+DSS only group with * indicating p < 0.05; ** p < 0.01; and *** p < 0.005. Representative sections of each group were taken at 400 x magnification and positive staining is brown colored.

Figure 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
week later, either given water *ad libitum*, or 2% DSS in the drinking water for 14 days as described in methods and in Figure 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 only had activation of iNOS in both CD45+ and CD45-cells. Mice consuming Cl-amidine + 2% DSS had iNOS attenuated in both cell types. (B) Following 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 methods, and expressed as mean (± SE) relative light units (RLU) per 1 x 10^6 cells. *, indicates a 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 (± SE) tail moment from 200 Comets taken from 4 mice per group. *, indicates a significant difference from the AOM+DSS only group (p < 0.01). Representative Comets for each group are shown.
Figure 2

A.

AOM

-7

0

7

14

Time (days)

 +/- 2% DSS

 +/- Cl-amidine

in drinking water

Harvest Colon

B.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Histology Score</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>AOM + DSS</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>AOM + DSS + 0.25 mg/ml Cl-amidine</td>
<td>*</td>
<td>6</td>
</tr>
<tr>
<td>AOM + DSS + 0.5 mg/ml Cl-amidine</td>
<td>*</td>
<td>6</td>
</tr>
</tbody>
</table>

% Weight change (mean ± SE) (throughout experiment)

- AOM: 7.0 ± 1.2 g
- AOM + DSS: 3.3 ± 1.3 g
- AOM + DSS + 0.25 mg/ml Cl-amidine: 8.3 ± 1.6 g
- AOM + DSS + 0.5 mg/ml Cl-amidine: 11.0 ± 1.4 g

Colon length (mean ± SE) (end of experiment)

- AOM: 8.7 ± 0.2 cm
- AOM + DSS: 7.7 ± 0.3 cm
- AOM + DSS + 0.25 mg/ml Cl-amidine: 9.0 ± 0.1 cm
- AOM + DSS + 0.5 mg/ml Cl-amidine: 8.6 ± 0.3 cm
Figure 3

A. 

![Bar chart showing IRS values for different treatments](image)

- AOM
- AOM + DSS
- AOM + DSS + 0.25 mg/ml Cl-amidine
- AOM + DSS + 0.5 mg/ml Cl-amidine

B. 

- AOM
- AOM + DSS
- AOM + DSS + 0.25 mg/ml Cl-amidine
- AOM + DSS + 0.5 mg/ml Cl-amidine
Figure 4

A. 

<table>
<thead>
<tr>
<th>IFNγ (Hrs.)</th>
<th>Vehicle</th>
<th>CI-amidine (10 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
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iNOS

Cox-2

GAPDH

B. 

Luminescence (% of no Cl-amidine control)

Hrs. after stimulation with IFN-γ

-12 0 1 2 3

No Cl-Amidine

10 µg/ml Cl-amidine

C. 

% DNA Damage

Time of coculture with activated macrophages (Hrs.)

0 4

No CI-Amidine

10 µg/ml CI-amidine
Figure 5

A. Doses of CI-amidine

<table>
<thead>
<tr>
<th>µg/ml</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>2.4</td>
<td>12</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

catalase
GPx1
SOD1
GAPDH

B. Average I.R.S. of Catalase

- AOM: n = 6
- AOM + DSS: n = 5
- AOM + DSS + 0.25mg/ml CI-amidine: n = 6
- AOM + DSS + 0.5mg/ml CI-amidine: n = 6

C. Average I.R.S. of GPx1

- AOM: n = 6
- AOM + DSS: n = 5
- AOM + DSS + 0.25mg/ml CI-amidine: n = 6
- AOM + DSS + 0.5mg/ml CI-amidine: n = 6

D. Average I.R.S. of SOD1

- AOM: n = 6
- AOM + DSS: n = 5
- AOM + DSS + 0.25mg/ml CI-amidine: n = 6
- AOM + DSS + 0.5mg/ml CI-amidine: n = 6
Figure 6

A.

<table>
<thead>
<tr>
<th></th>
<th>CD45+</th>
<th>CD45-</th>
<th>Pure p53</th>
<th>Activated ANA-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM + DSS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl-amidine (0.25 mg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl-amidine (0.5 mg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

iNOS

GAPDH

B.

![Luminescence (RLU/10^6 cells)]

AOM  AOM + DSS  AOM + DSS + 0.25 mg/ml Cl-amidine  AOM + DSS + 0.5 mg/ml Cl-amidine

C.

![Tail Moment]

AOM  AOM + DSS  AOM + DSS + 0.25 mg/ml Cl-amidine  AOM + DSS + 0.5 mg/ml Cl-amidine