Sepiapterin ameliorates chemically-induced murine colitis and azoxymethane-induced colon cancer.

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Abbreviations: AOM, azoxymethane; BH2, dihydrobiopterin; BH4, tetrahydrobiopterin; CRC, colorectal cancer; DSS, dextran sodium sulfate; NOS, nitric oxide synthase; PKG, cGMP dependent protein kinase G; RNS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble guanylate cyclase. SP, sepiapterin

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Abstract

The effects of modulating tetrahydrobiopterin (BH4) levels with a metabolic precursor, sepiapterin (SP), on dextran sodium sulfate (DSS) induced colitis and azoxymethane (AOM) induced colorectal cancer were studied. SP in the drinking water blocks DSS-induced colitis measured as decreased disease activity index (DAI), morphological criteria and recovery of Ca^{2+}-induced contractility responses lost as a consequence of DSS treatment. SP reduces inflammatory responses measured as decreased numbers of infiltrating inflammatory macrophages and neutrophils and decreased expression of pro-inflammatory cytokines IL-1β, IL-6 and IL-17A. HPLC analyses of colonic BH4 and its oxidized derivative, dihydrobiopterin (BH2), are inconclusive although there is a trend for lower BH4:BH2 with DSS treatment that was reversed with SP. Reduction of colonic cGMP levels by DSS is reversed with SP by a mechanism sensitive to 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a specific inhibitor of the NO-sensitive soluble guanylate cyclase (sGC). ODQ abrogates the protective effects of SP on colitis. This plus the finding that SP reduces DSS enhanced protein Tyr nitration are consistent with DSS induced uncoupling of NOS. The results agree with previous studies demonstrating inactivation of sGC in DSS-treated animals as being important in recruitment of inflammatory cells and in altered cholinergic signaling and colon motility. SP also reduces the number of colon tumors in the DSS/AOM treated mice from 7 to 1 per unit colon length. Thus pharmacological modulation of BH4 with currently available drugs may provide a mechanism for alleviating some forms of colitis and potentially minimizing the potential for colorectal cancer in patients with colitis.
Introduction

The link between inflammation and cancer was proposed 150 years ago when Virchow argued that cancers arise at sites of chronic inflammation (Balkwill and Mantovani, 2001). The underlying mechanisms involve the recruitment of inflammatory cells and the generation of cytokines and enzymes that facilitate the development of the inflammatory microenvironment of the tumor and which are associated with other inflammatory diseases such as colitis. Important roles reactive oxygen/nitrogen species (ROS/RNS), in tumor initiation and progression are also clearly established (Wink et al., 1998; Westbrook and Schiestl, 2010).

Expression of inducible NOS (NOS-2) in both epithelial and invading inflammatory cells is enhanced in colitis contributing to nitro-oxidative stress throughout carcinogenesis and providing one explanation for the enhanced protein Tyr nitration observed in these CRC tissues (Itzkowitz and Yio, 2004). Of potential significance to colitis is the nitration and inhibition of the Ca\textsubscript{2+}1.2 calcium channel of colonic smooth muscle and its role in motility changes resulting from colitis (Ross et al., 2007; Kang et al., 2010). However, equally elevated levels of protein nitration are observed in chemically induced colitis of wild type and NOS-2 knockout mice and no difference in CRC incidence is observed in comparing these mice (Seril et al., 2007). One explanation is a compensatory mechanism involving increased expression of other NOS isoforms, e.g. the endothelial NOS (NOS-3).

For all NOS isoforms, a transfer of electrons is coupled with the oxidation of arginine by a mechanism in which the cofactor tetrahydrobiopterin (BH\textsubscript{4}) donates electrons to the NOS.
ferrous dioxygen complex to initiate oxidation. The concentration ratio of BH4 to its oxidation product 7,8-dihydrobiopterin (BH2) is critical since BH2 binds with equal affinity for NOS but in a catalytically non-productive manner. When the BH4:BH2 ratio is much greater than one, NOS catalysis generates NO and citrulline and NOS is said to be “coupled”. The primary target of NO is soluble guanylate cyclase (sGC), stimulate of cGMP synthesis, and activation of protein kinase G (PKG). This pathway is important in smooth muscle relaxation and synaptic transmission. In the vasculature, the NO-sGC-PKG pathway is anti-inflammatory. In colon carcinoma cell lines, increased PKG expression reduces cell proliferation and enhances apoptosis (Deguchi et al., 2005; Cen et al., 2008; Kwon et al., 2010).

When the BH4:BH2 ratio is low, as in chronic inflammatory conditions, coupling is less efficient and superoxide (O_2^-) is produced rather than NO. Under these conditions NOS is “uncoupled” and has been called “peroxynitrite synthase (Stuehr et al., 2001; Mikkelsen and Wardman, 2003; Alp and Channon, 2004; Cai et al., 2005). Peroxynitrite is a Tyr nitrating free radical. NOS uncoupling is extensively investigated in vascular diseases and a synthetic BH4 (Kuvan) is currently in clinical trials for these diseases and approved for use in the treatment of some forms of phenylketouria (Forstermann, 2010; Schmidt et al., 2010).

A previous study demonstrated that dietary supplementation with nitrite ameliorated DSS-induced colitis possibly by bypassing NOS activity and utilizing an alternative mechanism of NO generation involving the reduction of nitrite (Lundberg and Weitzberg, 2009; Ohtake et al., 2010). The goal of the present study is to examine the effect of modulating BH4 levels by
dietary pretreatment of animals with a metabolic precursor of BH4, sepiapterin (SP), thereby enhancing the NO-SGC-PKG pathway and abrogating DSS induction of colitis and CRC.

**Methods**

**Mouse model of colitis:**

Colitis was induced by the addition of 2.5% dextran sodium sulfate (DSS; USB Corporation, Cleveland OH) to the drinking water of 5-6-week-old C57 BL/6 male mice for a period of 7 days. Mice designated for SP (Schircks Laboratories, Switzerland) treatment received 4mg/100ml SP in their drinking water continually starting 3 days before DSS treatment. Drinking water was delivered with calibrated liquid feeding tubes from Bioserv. None of the supplements affected the amount of water consumed by the animals, on average about 4 mls per day. The dose of SP, approximately 0.64 mg/kg/day, is significantly less than used in previous studies on vascular function whether provided in food pellets (Pannirselvam et al., 2002) or in the drinking water (Shimazu et al., 2011). This dose was selected on the basis of preliminary experiments demonstrating that this dose increased BH4:BH2 levels in and was cytotoxic to different tumor cell lines grown as xenografts or in tissue culture (Rabender and Mikkelsen, in preparation). All animals were monitored daily for weight, stool consistency and the presence of blood in the excreta (Sasaki et al., 2005).

Disease activity index (DAI) was determined by combining scores for weight loss, stool consistency and bloody excreta as follows: Weight loss (0: <1%, 1: 1-5%, 2: 5-10%, 3:10-15%, 4: >15%); Stool consistency (0: normal, 2: loose, 4: diarrhea); Blood in excreta (0: normal; 2: reddish, 4: bloody). Long-term colitis/carcinogenesis was induced by the I.P. injection of AOM.
(10mg/kg) seven days prior to the onset of DSS treatments; three week long courses of DSS were given as above separated by 2 weeks of DSS-free water. Animals were sacrificed 3 weeks after the final DSS treatment, 77 days after AOM treatment. Mice designated for SP treatment received 4mg/100ml SP in their drinking water continually starting 3 days before DSS treatment. To inhibit sGC, animals were injected ip daily with 20 mg/kg ODQ as described in (Tseng et al., 2011).

All animals were monitored daily for weight, stool consistency and the presence of blood in the excreta. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and conformed to the guidelines established by the National Institutes of Health, protocol numbers AM10080 and AM10185.

**BH4/BH2 analysis:**

The purification and analysis of colonic biopterins was a modification of a previously described method (Fukushima and Nixon, 1980; Sawabe et al., 2004). The frozen distal section of colons was homogenized in 10 volumes 0.1N HCl and centrifuged at 13,200rpm for 20 minutes at 4°C. Aliquots of supernatant were mixed with a 0.625 volumes of an acid-iodine solution (2% I₂, 3% KI in 0.1N HCl) or alkaline-iodine (2% I₂, 3% KI in 0.2N NaOH) for oxidation under acid or alkaline conditions respectively (1 hour in the dark at room temperature). After adding 0.5 volumes of 2.5% ascorbic acid in 0.4N perchloric acid a clear solution was obtained by centrifugation for 10 minutes at 10,000 rpm. BH4 and BH2 were measured by HPLC using a fluorescence detector (Ex = 350nm, Em = 450nm). The solid phase was
Partisphere RTF C18 4.6x250mm (Whatman, Clifton, NJ) and the mobile phase was 7% methanol. BH4 and BH2 were quantified as described elsewhere (Sawabe et al., 2004).

**cGMP and MPO ELISA:**

For cGMP, frozen colons were homogenized in 10 volumes 0.1N HCl and centrifuged at 13,200rpm for 20 minutes at 4°C. The resulting supernatant was analyzed for cGMP using the cyclic GMP EIA Kit (Cayman Chemical Co.) following the manufacturer’s instructions. For analysis of MPO, colons were homogenized in the non-denaturing cell lysis buffer from Cell Signaling and MPO levels measured with the Raybiotech ELISA kit for mouse MPO.

**Isometric Tension Recording:**

Approximately 1.5-cm strips of distal colon were suspended in the longitudinal direction in an organ bath containing 15 ml of Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11 mM glucose, and 2.5 mM CaCl₂), bubbled continuously with carbogen (95% O₂ and 5% CO₂) at 37°C under a resting tension of 1 g and equilibrated for a period of 1 h. Isometric contractions were recorded by a force transducer (model GR-FT03; Radnoti, Monrovia, CA) connected to a personal computer using Acqknowledge 382 software program (BIOPAC Systems, Santa Barbara, CA). After equilibration in Krebs solution, tissues were incubated for 30 min in Ca²⁺-free high-potassium solution (80 mM) in which equimolar NaCl was replaced by KCl containing 0.1 mM EGTA and changed every 15 min. Cumulative dose-dependent contraction responses to CaCl₂ (10 μM to 10 mM) were performed in distal colon strips depolarized by Calcium-free high-K⁺ (80 mM)-physiological saline solution (without EGTA). The cumulative concentration–dependent...
increases in tissue tension by CaCl$_2$ were analyzed among tissue strips isolated from control, DSS, DSS with SP and SP alone treated groups of mice.

**Immunohistopathology**

Colons were excised from animals, flushed with PBS, cut longitudinally, rolled into “Swiss rolls” and immediately flash frozen in liquid N$_2$. Frozen Swiss rolls were embedded in Tissue-Tek OCT freezing medium and cryosections prepared.

For macrophage detection, frozen sections were fixed in ice-cold acetone for 10 min. After donkey serum blocking for 60 min at RT, the sections were stained with primary mAb for F4/80 (5 μg/mL, AbD Serotec) overnight at 4°C, followed by incubation with Alexa 594-labeled donkey anti-rat secondary IgG (2 μg/mL, Invitrogen) at RT for 1 hour. A negative staining control was performed by incubation with isotype control Abs. The coverslips were mounted using Vectashield mounting medium (Vector Laboratories). Images were captured using the Ariol Digital Pathology Platform (Leica Microsystems, Buffalo Grove, IL). Protein nitroTyr staining of 6 μm frozen, OCT embedded tissues was analyzed by immunofluorescence microscopy with mouse biotin labeled anti-nitroTyr (Cayman Chemical Co.) using a biotin-avidin kit for mouse primary antibodies from Vector Laboratories.

For Hematoxylin and Eosin staining, cryosections (6 μm) were fixed in 4% paraformaldehyde, submerged in Modified Mayer’s Hematoxylin, destained in acid ethanol, stained with eosin, dehydrated and mounted with permount. Images were captured and analyzed using the Ariol Digital Pathology Platform.
Gene expression analysis

Colons were excised, flushed with PBS and flash frozen using liquid N$_2$. The Qiagen RNase Mini Kit was used per the manufacturer’s instructions to extract mRNA. cDNAs were generated using the Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR per the manufacturer’s instructions using 1ug total RNA per reaction and Oligo(dT)$_{20}$ primers. For quantitative PCR analysis, transcription profiles of $il17a$, $il1b$, and $il6$ were assessed on an ABI prism 7900HT Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and FAM-labeled probe sets were obtained as pre-developed assay reagents from Applied Biosystems: $il17a$, Mm00439618_m1, $il1b$, Mm01336189_m1, $il6$, Mm99999064_m1. The PCR was started with 2 min at 50 °C and an initial 10 min denaturation at 94 °C, followed by a total of 40 cycles of 15 sec denaturation at 94 °C, and 1 min of annealing and elongation at 60 °C. All measurements were performed in triplicate wells and repeated three times. Gene expression was quantified relative to the expression of the housekeeping gene $b$-actin, and normalized to that measured in control cells by standard $2^{(-\Delta\DeltaCT)}$ calculation.

Statistics:

For contraction measurements the data from different groups were analyzed by two way-ANOVA followed by Bonferroni’s post-test. $P \leq 0.05$ was considered significant. For all other experiments, data are shown as mean +/-SEM with $P$ values calculated by student’s t-test or in the case of the microscopic analysis by a one way analysis of variance with each treatment group
as the only effect. The difference of least square means for each group was tested using a F test for statistical significance.

**Results**

**SP treatment ameliorates DSS-induced colitis.**

To determine the effect of SP upon experimental colitis animals were placed in four groups drinking water doped with DSS, SP, DSS and SP or untreated water and were monitored for the development of colitis. Colitis was measured by daily recordings of weight, stool consistency, and the presence of blood in the excreta. As shown in Figure 1A, by day 7 animals receiving DSS have lost 14% of their starting body weight. The weight loss in DSS treated animals is prevented by the addition of SP to the drinking water. Similarly, the disease activity index (Figure 1B) is significantly increased in only the DSS treated animals at day 7.

The development of DSS-induced colitis and its partial mitigation by co-treatment with SP was also monitored by H&E staining of frozen sections of colons from treated animals. As shown in Figure 2, a loss of villi and crypts is observed in the DSS alone treated colon and co-treatment with SP mostly mitigates this change in colonic morphology. Separation of the muscle and *muscularis mucosae* is predominantly observed in both the DSS-treated colons but was also observed to some degree in SP colons indicating mild colitis in DSS with SP colons that is not detected by assessment of the disease activity index.
SP rescues the reduced calcium-induced contraction in murine colitis.

Cumulative addition of CaCl₂ induced a concentration-dependent contraction in both control and inflamed (DSS treated) distal colon depolarized by Ca²⁺-free high potassium (80 mM) physiological saline solution. DSS treatment significantly (P ≤ 0.01) reduced the overall calcium-induced contraction curve (Figure 3A, B & C), as analyzed by Two-Way ANOVA (F(1,28)=8.30). In vivo treatment of SP reversed the reduced CaCl₂-contraction in the DSS treated group (Figure 3B & E) without affecting the contractility in the control group (Figure 3D) (DSS plus SP, F(1,28)=26.65; SP, F(1,28)=0.42, Two-way ANOVA, n=3 per group). The efficacy of CaCl₂ is significantly (P ≤ 0.001) enhanced by SP treatment in the DSS treated group, unlike in otherwise untreated animals.

The DSS-induced inflammatory response is reduced by SP treatment.

DSS treatment is known to elicit an inflammatory response that includes the recruitment of infiltrating immune cells such as macrophages. As SP treatment is expected to be anti-inflammatory we stained colon sections for the presence of macrophages. The top row of images of Figure 4 (F4/80 plus DAPI) show that infiltration of inflammatory macrophages is observed throughout the length of the mouse colon after DSS treatment and this is mostly reversed by inclusion of SP in the drinking water of mice. Higher magnifications are also shown in the lower rows of images. Statistical significance was established by semi-quantitative analysis of the images of 3 images from 4 mice in each treatment group as shown in Supplemental Data Figure 1A. In addition measurements of myeloperoxidase protein by ELISA demonstrated elevated
levels of neutrophils after DSS treatment and reversible with co-administration of SP (Supplemental Data Figure 1B). These results confirm that DSS treatment elicits an inflammatory response and that it is at least partially reversible with SP.

To determine how SP treatment reduced macrophage recruitment in DSS-treated colons, gene expression analysis was performed to determine the expression of pro- and anti-inflammatory cytokines. As shown in Figure 5, DSS treatment elicited significant increases in the expression of mRNA for the pro-inflammatory cytokines IL-1β, IL-6 and IL-17A. This increased cytokine expression is blocked by the addition of SP to the drinking water. We also assayed for the anti-inflammatory cytokine IL-37 since this cytokine has been shown to protect the mouse colon from DSS induced colitis (McNamee et al., 2011). However, IL-37 expression was not detected in any of the colon samples tested. The observed changes in cytokine expression indicate that SP acts by disrupting both the acute phase response and delayed-type immune reaction.

**SP blocks the DSS induced reduction in colon cGMP levels.**

To determine if DSS induced inflammation decreases NOS coupling and thus NO production we assayed the BH4:BH2 ratio following 7 days DSS treatment. Colonic cGMP levels were also measured at this time as an indirect measure of coupled NOS activity. Figure 6 in Supplemental Data shows the results from one experiment where the BH4:BH2 was measured at day 7 of DSS treatment. While the trend of lower BH4:BH2 with DSS treatment was observed the magnitude of the change never reached statistical significance due to the high
variability between samples. SP treatment with or without DSS, however, increased the BH4:BH2.

While we were unable to observe any significant change in the BH4/BH2 ratio with DSS treatment, production of cGMP – an indirect measure of NO production – was significantly reduced in DSS treated colons (Figure 7B, P<0.01). The DSS-induced reduction in levels of cGMP was reversed by SP treatment. SP alone significantly increased cGMP production relative to control animals.

cGMP is also generated by a NO-independent but Ca\textsuperscript{2+}-requiring membrane bound guanylate cyclase. That SP treatment reverses the effect of DSS on colon cGMP levels suggests that increased NO generation is required. To further test this mechanism we used the sGC inhibitor, ODQ, which is an irreversible inhibitor of sGC. In these experiments, ODQ was delivered ip on a daily basis with changes in the SP-containing water. As shown in Figure 7B, ODQ blocked the activity of SP to prevent the decrease in cellular cGMP in the colons of mice treated with DSS. Parallel experiments were performed in which the DAI of the animals were measured. As shown in Figure 7A, ODQ also prevented the SP-induced recovery in the DAI of DSS treated animals.

**SP inhibits DSS-induced protein Tyr nitration.**

Previous investigators have termed uncoupled NOS as a “peroxynitrite synthase”. Thus if uncoupling of NOS occurs with DSS treatment one would expect increased protein Tyr nitration as has been observed in numerous studies with DSS-induced colitis, e.g. (Yasui et al.,
As shown in Figure 8, DSS treatment enhances colonic protein Tyr nitration as detected by immunofluorescence and this was abrogated by co-administration of SP. Statistical analysis demonstrated a >5-fold increase in nitroTyr staining relative to DAPI fluorescence in the colons from DSS treated animals compared to controls and SP+DSS treated animals (p<0.01, n=3 animals per treatment group and 3 images per animal). As shown in image N, a magnification of image J, the increased nitroTyr staining was due not only to infiltrating inflammatory cells but also colonic epithelial cells. This result along with the observed changes in cGMP and their reversibility by treatment with the BH4 precursor, SP, are consistent with uncoupling of NOS in DSS-induced colitis.

**SP partially protects against AOM/DSS induced adenocarcinoma.**

Given that SP protects against the development of DSS-induced colitis we tested the hypothesis that this protection may extend to preventing the development of AOM/DSS induced CRC. Animals received a dose of AOM followed by three one-week courses of DSS with or without SP co-treatment. Following 70-days of treatment, colons were excised and assayed morphologically for tumor development. Individual single treatments with AOM or DSS did not induce cancer formation as previously described (Neufert et al., 2007). As seen in Figure 9A, the combined AOM+DSS treatment induced tumor formation that was significantly reduced by co-treatment with SP (P<0.05). In the ten animals treated with AOM+DSS as described, the number of tumors per distal colon ranged between 2 and 8. The ten animals in the AOM+DSS+SP group the number of tumors per colon ranged between 0 and 3. The modest increase (15%) in colon length with SP treatment was insufficient to account for the difference.
observed between the two groups. Interestingly, the SP co-treatment did not decrease the average size of those tumors that do develop (Figure 9B) suggesting that SP acts to limit tumor initiation rather than restricting tumor progression. However, tumors were harvested at a relatively late time post-initiation and effects of SP on tumor progression would have been missed.

Discussion

By multiple criteria, we have demonstrated that co-treatment of animals with SP counteracts the colitis inducing effects of DSS. Thus SP treatment blocks the increased DAI, the loss of microvilli and crypts, and recovers the Ca\(^{2+}\)-induced contractility responses lost as a consequence of DSS treatment. In addition the experimentation demonstrates that SP treatment reduces the inflammatory response measured as either increased numbers of infiltrating inflammatory macrophages and neutrophils or increased expression of pro-inflammatory cytokines IL-1β, IL-6 and IL-17A. Our studies also show that by minimizing inflammation SP also decreased the numbers of tumors formed as a consequence of tumor initiation with the carcinogen AOM.

The initial hypothesis for this study was that DSS treatment would further promote the chronic inflammatory environment characteristic of colitis by reducing the BH4:BH2 ratio and uncoupling NOS activity resulting in the generation of ROS/RNS. However, the observed changes in the colonic BH4:BH2 ratio while consistent with the hypothesis were not statistically significant. Although a significant decrease in the BH4:BH2 was not measured in the colons from DSS-treated animals, the observed changes in cGMP levels and protein Tyr nitration
following DSS treatment and their reversibility with SP are consistent with NOS uncoupling. A previous study demonstrated that DSS-induced colitis is associated with inhibition of colonic sGC activity leading to lower cGMP levels (Van Crombruggen et al., 2008; Schmidt et al., 2012). The underlying mechanism for the inhibition was not established but it did not involve changes in either nNOS mRNA or protein levels. These authors argued that the reduced sGC activity was associated with a decreased colonic responsiveness to nitrergic stimuli. More recently a mechanism for the inactivation of sGC in inflammatory conditions has been shown to involve oxidation of the heme cofactor of sGC (Schmidt, 2012 #9253). Protection of sGC from oxidative inactivation \textit{in vitro} was achieved by culturing cells with BH4 or its precursors BH2 and SP. This latter mechanism can occur without changes in the BH4:BH2 and NOS coupling.

Measurements of total BH4:BH2 in the whole distal colon do not necessarily reflect changes in the different cellular compartments of the colon that may have an impact on colitis. For example, DSS-induced NOS uncoupling in nitrergic neurons by disrupting the normal nicotinic-nitrergic communication could by inhibiting cholinergic signaling increase secretion relative to absorption and thereby stimulate diarrhea (Green, 2004 #9183). The immunofluorescent images of protein Tyr nitration also suggest localized changes in NOS activity with DSS treatment.

Colitis is a consequence of the relative changes in secretory and absorptive functions of the colon that results in changes in gastrointestinal motility. Genetic, environmental, microbial and immunological factors all play a role ultimately resulting in loss of barrier function and the pathological invasion of inflammatory cells into the mucosa and increased expression of
inflammatory cytokines. It remains unclear how these different factors relate to one another in initiation and progression of colitis and where NO signaling and biopterins such as BH4 and SP contribute to the development and prevention of colitis. One mechanism that deserves consideration also involves sGC and its role in inhibiting the recruitment of inflammatory cells to sites of tissue injury (Ahluwalia et al., 2004). In endothelial cells sGC activity inhibits inflammatory cell recruitment by blocking P-selection expression (Ahluwalia et al., 2004). Thus besides restoring maintaining barrier function and the normal balance of secretion and absorption, SP by protecting sGC from oxidative inactivation may also mitigate the effects of DSS-induced colitis by preventing the recruitment of inflammatory cells.

The three cytokines examined, IL-1β, IL-6, and IL-17 have all been associated with DSS induced colitis and with colon CRC (Kaler et al., 2009; Hyun et al., 2010; Waldner et al., 2012). One unifying feature for all three cytokines is their transcriptional regulation by NF-κB (Funakoshi et al., 2012). A relatively specific inhibitor of NF-κB, dehydroxymethylepoxyquinomicin, inhibits the mRNA expression of IL-β, IL-6, TNF-α, IL-12p40, IL-17A and MCP-1 and also suppresses both DSS and TNBS-induced colitis. With respect to how SP inhibits pro-inflammatory cytokine expression one mechanism is based on a previous study showing that low inflammatory levels of RNS result in the nitration Tyr181 of IκBα, the inhibitor protein of NF-κB, dissociating the complex and facilitating translocation of active transcription factor, p65/p50, into the nucleus (Yakovlev et al., 2007). SP treatment by increasing the BH4:BH2 recouples NOS activity enhancing NO generation and reducing RNS generation necessary for Tyr nitration. Furthermore, NO dependent S-nitrosylation of p65 also
JPET #203828 inhibits NF-κB transcriptional activity (Marshall et al., 2004). Thus recoupling of NOS potentially inhibits NF-κB activity leading to suppression of the inflammatory response critical for colitis and colitis associated carcinogenesis (Sawabe et al., 2004; Atreya et al., 2008; Shaked et al., 2012). Regardless of the mechanism of SP activity these results suggest a potential approach in mitigating colitis and associated CRC by modulating colonic BH4/cGMP metabolism. One potential candidate is a synthetic BH4, Kuvan, currently used for treatment of some types of phenylketonuria. Kuvan is also being examined in Phase 2 trials for treatment of different inflammatory and vascular diseases (Burton et al., 2011; Cunningham et al., 2012).

Author Contributions

Participated in research design: Cardnell, Rabender, Akbarali, Wang, Mikkelsen.
Conducted experiments: Cardnell, Rabender, Alam, Howlett, Ross, Guo.
Performed data analysis: Cardnell, Rabender, Wang, Ross, Guo, Mikkelsen
Wrote or contributed to the writing of the manuscript: Cardnell, Rabender, Akbarali, Wang, Mikkelsen
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Footnotes

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Legends for Figures

Figure 1. Disease activity in mice with experimental colitis. Disease activity in animals receiving 2.5% DSS in their drinking water was significantly reduced in animals that also received 4mg/100ml SP in their drinking water. Attenuated disease was observed as measured both by animal weight (A) and disease activity index (B). Data presented as mean ± SEM, n=5 per group.

Figure 2. H&E staining of colons from control and treated mice. H&E stained frozen sections of colon “swiss rolls”. Swiss roll preparations allow the visualization of the whole colon distal (D) to proximal (P) in a single section.

Figure 3. Colon contractility in DSS-induced colitis. Representative isometric tension recording of CaCl$_2$–induced concentration–dependent contraction of distal colon tissue strips from A) control, B) DSS and DSS with SP treated mice. Right panel displays graphical representation and comparison of concentration–dependent CaCl$_2$–induced increase in tension of colon muscle strips: C) control vs DSS, D) Control vs SP, and E) DSS vs DSS and SP. Note that inflammation decreased the contraction while treatment with SP increased the CaCl$_2$–induced contraction in inflamed tissues. Data are expressed as mean ± SEM, and analyzed by Two-Way ANOVA followed by Bonferroni post-test, n=3 per group.

Figure 4. SP treatment decreases macrophage and neutrophil colonic infiltration into DSS-treated animals. A. Cryosections of colon were stained with rat anti-mouse mAbs for F4/80, followed by incubation with Alexa 594-conjugated donkey anti-rat secondary IgG. Scale bar,
100 μm. B. Quantification of macrophage infiltration. Three images from each treatment set from three separate animals were randomly selected and total fluorescence in each due to F4/80 staining was quantified relative to DAPI fluorescence with the Ariol Immunohistopathology Work Station. The entire colon is shown as swiss rolls in the top row. Typical 20X magnification images are shown in the bottom rows. The bottom row shows the control images without the primary antibody. Statistical analysis is provided Supplemental data Figure 1.

**Figure 5. Increased pro-inflammatory cytokine expression of diseased colons is reduced by SP treatment.** Effect of SP co-treatment on *il1b*, *il6* and *il17a* gene expression levels in the diseased colons was assayed by quantitative real-time RT-PCR and normalized to the *b-actin* gene in the control group. Each bar represents the cDNA from one animal analyzed in triplicate with the error bars representing +/-SEM for the triplicate samples. & Not detectable.

**Figure 6. BH4: BH2 levels in colons from control and treated mice.** A. BH4 and BH2 were measured by HPLC analysis as described in Materials and Methods. Each bar represents the mean +/-SEM of four colons for each treatment.

**Figure 7. The sGC inhibitor, ODQ, inhibits the SP mitigation of DSS-colitis and blocks SP induced cGMP increases in DSS treated colons.** A. DAI was measured as described in Figure 1. B. cGMP assayed by ELISA shows decreased cGMP production in DSS-treated whole colons that is restored when animals also received SP. cGMP is an indirect measure indicating decreased NO production in DSS-treated colons. Data presented as mean ± SEM, 4 colons per treatment group with *P<0.05 for DSS and SP compared to Control or DSS+SP.
Figure 8. NitroTyr staining is increased in diseased colons by a mechanism inhibited by SP. Images A-F are DAPI+fluorescein-Avidin stained Swiss rolls with images G-L the corresponding fluorescein-Avidin stained sections with the blue DAPI fluorescence off. Lanes A+G (no primary anti-nitroTyr) and B+H (+primary anti-nitroTyr) are adjacent cryostat sections from the colons of untreated animals; C+I (no primary anti-nitroTyr) and D+J (+primary anti-nitroTyr) are adjacent colon sections from DSS treated animals; E+K (no primary anti-nitroTyr) and F+L (+primary anti-nitroTyr) are adjacent sections from the colons of DSS animals rescued with SP. Images M and N are magnifications of sections from the adjacent images I and J showing infiltration of inflammatory cells into the colon of DSS treated animals as well as increased nitroTyr staining of epithelial cells.

Figure 9. Reduced DSS/AOM induced colon tumorigenesis in SP treated animals. A. Number of tumors identified per unit length of colon in Swiss rolls. The results represent the mean plus SEM for n=10 for each treatment group with a *P<0.05. B. Relative size of tumors evaluated digitally from H&E stained sections.
Figure 1
Figure 2

A. Control

B. DSS

C. DSS + SP

D. SP
Figure 3

A. 

Control

10 μM 30 μM 100 μM 300 μM 1 mM 10 mM

CaCl₂

B. 

DSS

10 μM 30 μM 100 μM 300 μM 1 mM 3 mM 10 mM

DSS + SP

C.

Tension (g/mg tissue wt) vs. CaCl₂ [log (M)]

D.

Tension (g/mg tissue wt) vs. CaCl₂ [log (M)]

E.
Figure 4

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Figure 5

- IL-1β
- IL-6
- IL-17α

Relative Expression

C, DSS, DSS+SP, SP
Figure 6
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Figure 7

A. Disease Activity Index

B. cGMP (pmol/gm)

Control	DSS	DSS+SP	DSS+SP+ODQ	ODQ+SP+DSS	ODQ+SP
Figure 8
Figure 9 A and B

A. Tumors/Unit Length

B. Relative Tumor Volume

AOM +DSS

AOM+DSS+SP

AOM +DSS

AOM+DSS+SP