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Aspirin and Low Dose Nitric Oxide-Donating Aspirin Increase Life Span in a Lynch Syndrome Mouse Model

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) appear to be effective cancer chemopreventives. Previous cellular studies demonstrated that aspirin (acetylsalicylic acid: ASA) and nitric oxide-donating ASA (NO-ASA) suppressed microsatellite instability (MSI) in mismatch repair (MMR)-deficient cells linked to the common cancer predisposition syndrome hereditary non-polyposis colorectal cancer or Lynch syndrome (LS/HNPCC), at doses 300-3000-fold less than ASA. Using a mouse model that develops MMR-deficient intestinal tumors that appear pathologically identical to LS/HNPCC we show that ASA (400 mg/kg) and low dose NO-ASA (72 mg/kg) increased life span by 18–21%. We also note a trend where ASA treatment resulted in intestinal tumors with reduced high MSI (H-MSI) and increased low MSI (L-MSI) as defined by the Bethesda Criteria. Low dose NO-ASA had a minimal effect on MSI status. In contrast to previous studies, high dose NO-ASA (720/1500 mg/kg) treatments increased tumor burden, decreased life span and exacerbated MSI uniquely in the LS/HNPCC mouse model. These results suggest that MMR-deficient tissues/mice may be specifically sensitive to intrinsic pharmacokinetic features of this drug. It is likely that long-term treatment with ASA may represent a chemopreventive option for LS/HNPCC patients. Moreover, since low dose NO-ASA demonstrates equivalent life span increase at 10-fold lower doses than ASA, it may have the potential to significantly reduce the gastropathy associated with long-term ASA treatment.

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are a structurally diverse family of compounds that are effective in the prevention of colorectal cancer (1, 2). Acetylsalicylic acid (ASA), commonly known as aspirin, is the archetype of the NSAID family. Epidemiological studies have reported an inverse relationship between ASA use and the incidence of colorectal cancers (3, 4). Animal models have confirmed that administration of various NSAIDs results in fewer tumors (5, 6).

Nitric oxide-donating NSAIDs (NO-NSAIDs) are novel compounds in which a NO-donating group is attached to the NSAID through a linker molecule. The rationale is that the NO-moiety may diminish or alleviate undesirable NSAID-induced side-effects such as gastropathy (7, 8). NO-NSAIDs also share many pharmacological properties with their parental molecules and may possess greater efficacy as chemopreventive agents (7, 8). NO-ASA is the most potent NO-NSAID reported to date, demonstrating at least 100-fold more activity than other NO-NSAIDs in diverse experimental systems (9-12). It is also more effective than aspirin at reducing tumorigenesis in rodent models of cancer (13-16).

The DNA mismatch repair (MMR) pathway recognizes and repairs nucleotide mispairs generated by post-replication misincorporation and genetic recombination between heteroallelic DNAs, as well as several DNA damage-specific lesions (17, 18). There is compelling evidence that links mutations in the *MSH2*, *MSH6*, *MLH1* and *PMS2* genes with Lynch syndrome/hereditary non-polyposis colorectal cancer (LS/HNPCC) (19). Tumor tissues from most LS/HNPCC cases associated with MMR defects display microsatellite instability (MSI) (20). The mechanism of tumorigenesis has been linked to the mutator phenotype conferred by MSI, which provides the environment for the accumulation of multiple secondary mutations that drive tumorigenesis (21).

ASA suppresses the MSI mutator phenotype of MMR-deficient human colon tumor cell lines (22, 23) via a genetic selection that appears to enhance apoptosis in critically unstable cells. The long term outcome is a cell population that has a persistent deficiency in MMR but paradoxically has acquired a largely microsatellite stable (MSS) phenotype (22, 23). NO-ASA also suppresses MSI in MMR-deficient cell lines at concentrations 300-3000-fold less than ASA (23). It was anticipated that treatment with ASA and NO-ASA would delay and/or prevent tumorigenesis in LS/HNPCC animal models of intestinal cancer.

Here we examined the effect of ASA and NO-ASA in a mouse model of LS/HNPCC (*Msh2^{flox/flox}VpC^{+/+}*) that recapitulates the intestinal-specific disruption of *Msh2* function (24). ASA (400 mg/kg) and low dose NO-ASA (72 mg/kg) increased the life span of the LS/HNPCC mice. Increased survival by NO-ASA occurred at a tenfold lower equimolar dose than that by ASA. However, dietary exposure of mice to 720 mg/kg NO-ASA, an equimolar dose to 400 mg/kg ASA, as well as 1500 mg/kg NO-ASA accelerated intestinal tumorigenesis uniquely in MMR-deficient LS/HNPCC mice. This study suggests the long-term use of ASA in LS/HNPCC and should also enable reconsideration of NO-ASA structure such that higher doses, greater efficacy and reduced toxicity may be accomplished.

METHODS

Generation of mice

The development and subsequent characterization of the LS/HNPCC mouse model used in this study, herein designated *Msh2^{flox/flox}VpC^{+/+}*, has been reported elsewhere (24). The targeting strategy has been outlined in Supplementary Fig. S1. Mice were maintained on a mixed background of C57Bl/6J (~93%) and 129X1/SvJ (~7%). The following primers were used for genotyping at the floxed *Msh2* locus: 184F (TAC TGA TGC GGG TTG AAG G); 130F (TGT GCT GGC TCA CTT AGA CG); 165R (GGC AAA CTC CTC AAA TCA CG). Tissues containing the floxed *Msh2* allele yielded a PCR product of 984 bp, amplified by primers 130F and 165R. Cre-mediated *loxP* deletion of *Msh2* exon 12 was detected at the molecular level by PCR with primers 184F and 165R, which yielded a 341 bp product (Supplementary Fig. S2). The *Cre* transgene was genotyped with the following primers: IMR0015 (CAA ATG TTG CTT GTC TGG TG); IMR0016 (GTC AGT CGA GTG CAC AGT TT); IMR1878 (GTG TGG GAC AGA GAA CAA ACC); IMR1879 (ACA TCT TCA GGT TCT GCG GG). Primers IMR0015/IMR0016 amplified a 200 bp product, indicative of the endogenous wild-type allele. Primers IMR1878/IMR1879 yielded an 1100 bp product, indicative of the villin promoter-Cre (**VpC**) transgene. The *VpC* control mice were obtained from The Jackson Laboratories (strain B6.SJL-Tg(Vil-cre)997Gum/J) and were genotyped as above.

Animals were bred in a barrier one facility and were maintained according to the NIH animal care and use guidelines. All experiments involving animals received prior approval from the OSU Institutional Animal Care and Use Committee.

Drugs and treatment groups

Acetylsalicylic acid was obtained from Sigma. The *ortho* isomer of NO-ASA [2-(acetyloxy)benzoic acid 2-[(nitrooxy)methyl]phenyl ester; NCX-4060] was synthesized by the Indofine Chemical Company, Inc. Animals were fed a powdered diet from Harlan Laboratories, Teklad LM-485 (7912). Aspirin and NO-ASA were mixed with powdered food and administered *ad libitum*. Treatment was started after weaning when mice were around 25 days of age. Food was formulated on a weekly basis and any food remaining from the prior week was discarded.

Criteria for early removal

The *Msh2^{flox/flox}VpC^{+/+}* mice used in this study developed intestinal tumors spontaneously over their life time. Long term, this resulted in moribund animals that presented with a variety of tumor-related signs including weight loss; marked abdominal distension, possibly accompanied by a hunched posture and poor coat quality; dyspnea; possible enlarged swelling of the lymph nodes around the front and hind legs; anemia. Animals were inspected daily, in accordance with the guidelines required by the OSU Institutional Animal Care and Use Committee. Upon observation of these conditions, in addition to lethargy for >24 hours, unresponsiveness to stimuli, or anorexia for >24 hours, such mice were sacrificed and a complete pathological examination performed. Starting at 6 months, all animals were weighed weekly. Weight loss of >20% resulted in removal of the animals from the study for

subsequent pathology. If a consensus opinion could not be reached about a particular animal's health, the attending veterinarian was consulted.

Tumor analysis and histopathologic classification

All animals in the study were subject to criteria for early removal, and were euthanized by CO₂ asphyxiation, followed by cervical dislocation, for pathological examination before they died from tumor-associated complications. Mice were examined for the presence of tumors. Intestinal tumors were examined macroscopically and with a dissecting microscope. Tumor number and location were recorded. Tissues were fixed in 10% formalin, embedded in paraffin and 4 μ m sections were cut for slides. Sections were stained with hematoxylin and eosin according to standard protocols. A trained pathologist evaluated the slides.

PCR analysis of MSI

The mouse primers used for MSI analysis have been reported previously as TG₂₇, TA₂₇, GA₂₉, CT₂₅/CA₂₇, and A₃₃ (25). They have been re-designated herein as MSM01 to MSM05, respectively. One oligomer of each primer set was 5'-labeled with FAM, VIC or NED. Details for all primer pairs are presented in Supplementary Table S3. Genomic DNA was prepared from mouse tissues using a DNeasy tissue kit (Qiagen). DNA was amplified with Platinum *Pfx* DNA polymerase. The final components of the reaction mix were as follows: DNA 2–10 ng; amplification buffer (2x concentration); 1 mM MgSO₄; 300 μ M deoxynucleotide triphosphates; 300 nM of each primer; 1 unit of *Pfx*; and H₂O to a final volume of 15 μ l. The following amplification conditions were used as a standard: 95°C, 2 min; (94°C, 20 s; 58°C, 30 s; 68°C, 30 s) for 30 to 35 cycles; 68°C, 2 min. Samples were diluted and analyzed on an Applied Biosystems 3730 DNA analyzer. At least two independent amplification reactions of each microsatellite sequence were examined for each DNA sample. If there was any ambiguity about the outcome, the amplification was repeated a third time.

Small-pool PCR analysis of MSI was performed in a similar manner, except that ~100 pg of DNA was used as a starting template. Forty-eight reactions were performed on each DNA sample from matched sets (n=8) of ear, normal intestinal and tumor intestinal tissues from each of the treatment groups. The microsatellite profiles were transformed into distribution patterns similar to the examples presented in Fig. 4.

Comparative MSI analyses of intestinal tumors from LS/HNPCC mice

Comparative MSI was calculated by assessing changes at microsatellite loci between matched sets of normal intestinal and tumor tissues from mice in each treatment group. The relative degree of MSI in tumors was subsequently scored as follows: MSS, no comparative changes in microsatellite status; low microsatellite instability (MSI-L), MSI displayed in 1/5 (25%) microsatellite markers; high microsatellite instability (MSI-H), MSI displayed in at least 2/5 (>25%) markers (See Supplementary Table S2). We often found that one marker was largely uninformative. However, this criteria is identical to the NCI definition of MSI (20). Numbers are expressed as a percentage (in parenthesis) of the total number of tumors analyzed for each group. Fisher's exact test (two tailed) was used to calculate the

significance of comparative changes in MSI between MSI-L and MSI-H tumors from NSAID treated *Msh2^{flox/flox}VpC^{+/+}* mice.

Statistical analyses

All statistical analyses were generated with Graphpad Prism 5.0 software. Survival data from the Kaplan-Meier plots were compared with the log-rank test. Differences in tumor burdens were compared with the Mann-Whitney *U* test. Comparative MSI was evaluated with a two-tailed Fisher's exact test. In all cases a value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Dietary exposure to ASA and low dose NO-ASA increase survival of an LS/HNPCC mouse model

Only cells of the intestinal epithelia were specifically targeted for *Msh2* knock-out in our LS/HNPCC (*Msh2^{flox/flox}VpC^{+/+}*) mouse model (Supplementary Figs. S1–S3). *Msh2*-dependent tumorigenesis was confined to the intestines and had all of the pathological hallmarks of HNPCC tumors in humans (24). Groups of *Msh2^{flox/flox}VpC^{+/+}* mice were treated as follows: untreated, 400 mg/kg ASA, 72 mg/kg NO-ASA, 720 mg/kg NO-ASA, 1500 mg/kg NO-ASA. The 720 mg/kg NO-ASA dose is equimolar to that of 400 mg/kg ASA. Drugs were mixed with powdered diet and administered *ad libitum*. Administration of compounds by this method precluded an accurate estimation of the amount of food consumed per animal. Both ASA and NO-ASA, even at the higher doses appeared equally palatable to mice. Thus, there were no apparent issues associated with consumption of drug-laced feeds. We did not examine *Msh2^{flox/flox}* mice since in the absence of villin-Cre, these animals would be similar to wild type mice where tumors do not develop because of functional *Msh2* in the intestinal tissues (24). Groups of (*Msh2^{+/+}*)*VpC^{+/+}* mice were included to control for confounding effects of constitutive *Cre* expression in the intestine which could potentially cause tumorigenesis. One group was untreated; the other received 720 mg/kg NO-ASA. Kaplan-Meier survival plots were generated for mice in each group (Fig. 1).

Median survival times for LS/HNPCC mice were as follows: untreated (333 days); 400 mg/kg ASA (393 days), 72 mg/kg NO-ASA (403 days), 720 mg/kg NO-ASA (229 days); 1500 mg/kg NO-ASA (175 days). Pair-wise comparisons between groups were significant, with $P < 0.0001$ (log-rank test), except untreated mice versus 400 mg/kg ASA ($P = 0.0007$) and untreated mice versus 72 mg/kg NO-ASA ($P = 0.0003$). Unexpectedly, dietary exposure to 720 and 1500 mg/kg of NO-ASA significantly decreased survival of *Msh2^{flox/flox}VpC^{+/+}* mice in a dose dependent manner (Fig. 1). In contrast, the untreated and 720 mg/kg *VpC^{+/+}* control groups survived in excess of 800 days (Fig. 1) and their median survival times could not be calculated since they were eventually removed from the study to determine tissue MSI and tumor status, if any. Based on previous work (24), it is evident that the effects of high dose NO-ASA observed in our studies are genotype-specific; only manifesting in the LS/HNPCC mouse model but not wild type mice. These effects appear to be associated with

intrinsic pharmacokinetic features of the drug (discussed below). ASA and low-dose NO-ASA regimes increased the survival of LS/HNPCC mouse model by 18–21%.

ASA and NO-ASA treatment affect intestinal tumorigenesis in an LS/HNPCC mouse model

Tumors were confined to the intestines of the LS/HNPCC mice, predominantly in the duodenum and jejunum, and rarely in the ileum (Table 1). Although mice treated with ASA and low-dose NO-ASA lived longer and presented with tumors significantly later than untreated mice, they developed statistically equivalent numbers of tumors at the time of death (1.68 ± 0.72 and 1.78 ± 1.25 versus 1.90 ± 1.02 , respectively). Since the end-point of this study was survival, we cannot determine whether ASA and low-dose NO-ASA affect initiation and/or progression of the tumorigenic process in this mouse model.

In contrast, *Msh2^{fllox/fllox}VpC^{+/+}* LS/HNPCC mice treated with 720 mg/kg and 1500 mg/kg NO-ASA displayed a 3–4-fold increase in tumor number (5.10 ± 2.81 and 5.00 ± 1.73 tumors, respectively) (Fig. 2A; Table 1). These values are significant when compared to either the untreated, ASA treated, or 72 mg/kg NO-ASA treated mice ($P = 0.0002$, Mann-Whitney *U* test). Mice receiving 720 mg/kg NO-ASA developed as many tumors as those on 1500 mg/kg but the mean survival time of the latter group was only 76% that of the former (175 versus 229 days). Although more intestinal lesions and tumors developed in mice treated with high dose NO-ASA, similar types of lesions, including adenomas and adenocarcinomas, were identified in all *Msh2^{fllox/fllox}VpC^{+/+}* LS/HNPCC mouse model groups (Fig. 2B; Supplementary Table S1). Taken together with the survival analysis, these results suggest that high dose NO-ASA accelerates genotype-specific tumorigenesis in the LS/HNPCC mouse model.

Intestinal tissues from ASA and NO-ASA treated mice exhibit persistent MSI

To determine whether tissues from the drug-treated LS/HNPCC mouse model displayed MSI characteristic of MMR deficiency, DNA was isolated from ear (E), intestinal tumor (T) and adjacent normal (N) intestinal tissues. Analysis of MSS ear tissues (MMR-proficient) provided constitutional microsatellite profiles from which subsequent evaluations of corresponding normal and tumor intestinal instability were made. Five microsatellite markers, MSM01–05, were investigated for each matched set of three tissues. Representative examples of the MSI profiles are presented in Fig. 3. MSM02 has not been included, as it was only informative for 1/158 intestinal samples from *Msh2^{fllox/fllox}VpC^{+/+}* LS/HNPCC mice (Supplementary Table S2). The microsatellite profiles of both normal and tumor intestinal tissues remained unstable relative to the MSS patterns of the ear in ASA and NO-ASA treated mice (Fig. 3; Fig. 4; Supplementary Table S2). Intestinal tissues, either normal or tumor, from *Msh2^{fllox/fllox}VpC^{+/+}* LS/HNPCC mice displayed MSI, with 147/158 tissues showing differences at two or more microsatellite markers when compared to the ear samples. However, intestinal tissues and the occasional intestinal tumor which developed in untreated *VpC^{+/+}* mice or *VpC^{+/+}* mice treated with ASA were always MSS (Fig. 3A; Fig. 4A; Supplementary Table S2).

ASA may stabilize MSI in the intestinal epithelia of LS/HNPCC mice

Comparative MSI analysis provided a measure of the relative differences in instability between adjacent normal and tumor samples derived from the same tissues. We used the Bethesda Criteria to evaluate MSI status (20): MSS, no changes in microsatellite marker status; low MSI (MSI-L), MSI displayed in <40% microsatellite markers; high MSI (MSI-H), MSI displayed in >40% of the markers. Fisher's exact test was used to calculate the significance of comparative changes between MSI-L and MSI-H for tumors from NSAID treated *Msh2^{fllox/fllox}VpC^{+/+}* mice. MSI-L and MSI-H tumor numbers from untreated *Msh2^{fllox/fllox}VpC^{+/+}* mice were used as the baseline. Based on our previous cellular data we expected only a partial suppression of the MSI phenotype, which might cause a shift in the relative levels of MSI-H to MSI-L (22).

High dose NO-ASA induced relative increases in MSI where 94% of intestinal tissues from the 720 mg/kg and 86% of tissues from the 1500 mg/kg treated mice displayed MSI (Table 1). Moreover, 75% of tissues from mice receiving 720 mg/kg NO-ASA were characterized as MSI-H, compared to 33% of untreated. When the 1500 mg/kg MSI results are included, these data suggest a trend where high dose NO-ASA may exacerbate MSI in LS/HNPCC mice that correlates with the accelerated tumorigenesis. Treatment with low dose NO-ASA does not appear to aggravate or attenuate MSI in intestinal tissues of LS/HNPCC mice. In contrast, treatment with ASA appears to provoke partial stabilization of MSI in the intestinal epithelia. Untreated animals had a comparative MSI-L of 39% and MSI-H of 33% whereas ASA treated LS/HNPCC mice displayed an MSI-L of 56% and MSI-H of 22% (Table 2; Supplementary Table S2). These differences also did not attain statistical significance. However, there was a trend toward MSI stabilization by ASA that is consistent with the cellular studies (22, 23).

Additional high-resolution MSI analyses were performed by small-pool PCR with marker MSM01 (TG₂₇) on matched (E), (N) and (T) DNAs for a subset of mice in each treatment group. This enabled a more extensive assessment of the distribution of novel allelic variants at this locus in the microsatellite unstable normal and tumor intestinal tissues. The resulting profiles comprised a representational distribution of the intrinsic allelic diversity within each tissue (Fig. 4). Ear tissues from these animals were consistently MSS, with the major 137 bp allele always flanked by a relatively unvarying distribution of subsidiary alleles. Normal and intestinal tissues from LS/HNPCC mouse model in all five experimental groups demonstrated variation in both major allele sizes and allele distributions. Nevertheless, small-pool PCR analysis suggests that long-term treatment with ASA decreased both the distribution and number of allelic variants compared to untreated mice (Fig. 4). Taken as a whole, these observations appear to suggest that the underlying mechanism(s) through which ASA increases animal survival in our LS/HNPCC mouse model is by suppression of MSI. However, additional studies will be required to fully support this conclusion.

DISCUSSION

The role of NSAIDs in the prevention of colorectal cancer is mediated through various mechanisms, including the inhibition of cyclooxygenase 2 (COX-2), and the induction of apoptosis through COX-2 independent pathways (26). Previous studies from our laboratory

suggested that ASA partially suppresses the MSI-H phenotype of human colon cancer cell lines through an apoptotic mechanism that appeared to be COX independent (22). *In vitro* ASA has been shown to arrest colon cancer cells at the G₁/S checkpoint, and induce apoptosis through activation of ATM, p21 and BAX (27). Among NSAIDs, ASA is still a preferred choice for chemoprevention in average-risk individuals. This is not only because of its demonstrated chemopreventive efficacy, but also because of its unique potential in cardiovascular protection (3, 4).

In this study we investigated the chemopreventive potential of ASA and NO-ASA using a newly-developed mouse model of LS/HNPCC. LS/HNPCC mice treated with ASA at 400 mg/kg had an increased median survival time compared to untreated controls, with ~18% of the mice living to over 500 days (Fig. 1). Although ASA reduces tumor numbers in *Apc*^{Min/+} mouse models of colorectal cancer (28), and ASA may have a weak effect on *Apc*^{Min/+}*Msh2*^{-/-} mice treated *in utero* (29), this report is the first clear evidence that dietary aspirin increases survival in an LS/HNPCC mouse model (30).

An increase in survival of 18–21% could be described as modest. However this is still an important finding as it suggests that ASA should be considered as a chemopreventive alternative that may delay the rapid progression of tumors between clinical screenings of LS/HNPCC patients (31). Low dose NO-ASA (72 mg/kg) demonstrated similar efficacy toward increased lifespan at 10-fold less equimolar dose compared to ASA (Fig. 1). It is possible that this lowered dose could significantly reduce the gastropathies associated with long-term ASA administration.

ASA and low dose NO-ASA do not completely suppress tumorigenesis. This may be partially a result of our dosage structure, which for ASA was at the lower limit compared to previous colorectal carcinogenesis models (28, 29). Although LS/HNPCC mice treated with both drugs survive for longer than untreated controls, there was little difference in the eventual tumor numbers (Table 1). This indicates that prolonged NSAID treatment is unable to fully inhibit the inherent genomic instability of the MMR-deficient intestinal cells *in vivo*. It has been shown in a model of chronic colitis that inflammatory stimuli are sufficient to predispose *Msh2*^{-/-} mice to intestinal tumors (32). It is possible that inflammation increases in the intestinal compartment of LS/HNPCC over time, despite continual exposure to NSAIDs, or that the intrinsic MSI of the epithelia tissues eventually provides selective escape from aspirin-induced apoptosis in a sub-set of *Msh2*-deficient cells.

The available literature supports the chemopreventive superiority of NO-ASA over that of parental ASA (7, 8). It has consistently been demonstrated that animal models treated with NO-ASA can tolerate higher doses than ASA, and survive longer with less tumor burden (13-16). The decision to administer ASA at 400 mg/kg was based on an earlier study which demonstrated that similarly treated *Msh2*^{-/-} mice remained viable for up to a year without any obvious side-effects (29). 720 mg/kg NO-ASA is equimolar to 400 mg/kg ASA and enables a direct comparison of both compounds at the same effective concentration. These levels of NO-ASA enhanced tumorigenesis in our LS/HNPCC mouse model in a dose-dependent manner. Even though there were significant differences in survival and tumor burden between ASA treated and 720/1500 mg/kg NO-ASA treated *Msh2*^{flox/flox}*VpC*^{+/+} LS/

HNPCC mice, there were no notable disparities in the histologic classifications of intestinal lesions (Fig. 2B; Supplementary Table S2). These results suggest that treatment with high dose NO-ASA did not cause a shift in tumor origin or type, but merely accelerated incipient tumorigenesis. Moreover, the decreased survival caused by high dose NO-ASA treatment is genotype-specific for mice with the intestinal *Msh2* deletion.

Why does NO-ASA induce genotype-specific tumorigenesis *in vivo*? NO-ASA survives passage through the stomach intact and is subject to metabolic transformation only after absorption by the gut (33, 34). The regional increase in tumor formation is likely related to the uptake of NO-ASA and the subsequent appearance of quinone methide: an extremely reactive transient metabolite generated from the benzene linker (35-37). Quinone methides are highly reactive with nucleotides in DNA, among other biologically relevant molecules (38). The remarkably short lifetime of quinone methide intermediates complicates direct experimental assessment (35, 36).

It has been reported that quinone methides react with cellular pools of glutathione and thioredoxin, depleting their levels and contributing to oxidative stress (39, 40). Treatment of TK6 cells with NO-ASA resulted in H2AX phosphorylation and activation of ATM, indicative of double-strand breaks (41-43). NO-ASA also induced 8-oxoguanine lesions, indicative of increased levels of oxidative stress, in both LoVo and LRWZ cells (42, 44). Based on these observations we regard it likely that treatment with high-dose NO-ASA induced sustained levels of DNA damage, which may not be adequately repaired in an *Msh2*-deficient background. The absence of tumors in NO-ASA treated *wild-type* mice, as well as the lack of tumors originating at extra-intestinal *Msh2*-proficient tissues of our LS/HNPCC mouse model support this hypothesis.

Treatment with 72 mg/kg NO-ASA was initiated after it became apparent that 720/1500 mg/kg treatments induced tumorigenesis and following published reports of potential toxicity (16). These subsequent studies were performed to estimate a lower limit of toxicity in our LS/HNPCC mouse model. A regime of 72 mg/kg NO-ASA is equimolar to that of 40 mg/kg for ASA. To our knowledge, ASA has not demonstrated comparable efficacy when administered at such low doses on MSI or tumorigenesis in any animal model. It was completely unexpected that such a low NO-ASA dose would improve survival (Fig. 1). Like ASA, NO-ASA exhibits pleiotropic effects on cellular signaling pathways, which include induction of oxidative stress, inhibition of Wnt signaling, activation of the mitogen-activated protein kinase (MAPK) pathway, and inhibition of inducible nitric oxide synthase and inhibition of NF- κ B (45). This effect appears unique to the LS/HNPCC genotype and has led to studies that explore the specific effect(s) of the NO-linker on MSI, which should ultimately result in better NO-ASA agents.

A key prediction from earlier data was that both ASA and NO-ASA might attenuate MSI in the intestinal tissues of *Msh2*-deficient mice (22, 23). In fact, high dose NO-ASA treatment appeared to contribute to further instability as persistent MSI was detected in both normal and tumor intestinal tissues (Fig. 3E+F; Table 2). Small-pool PCR analysis of the same tissues supported this observation (Fig. 4E+F). Furthermore, despite promoting animal survival, low dose NO-ASA did not appear to have a significant effect on MSI in the

intestinal compartment. In contrast, ASA appeared to skew the total comparative MSI-H towards MSI-L when assessed in the context of the untreated control group (Fig. 4D; Table 2). These results are consistent with previous cellular data (22, 23) and support the conclusion that long-term dietary exposure to ASA may attenuate MSI in the intestinal tissues of *Msh2^{flox/flox}VpC^{+/+}* LS/HNPCC Mouse model. It is also possible that *in utero* treatment with ASA or NO-ASA may further reduce the onset of tumorigenesis and attenuate MSI in this mouse model.

One limitation of our current preclinical LS/HNPCC mouse model that is shared by essentially all mouse models of colorectal cancer is that tumors do not spontaneously develop in the colon (Table 1; 24); which contrasts the pathology observed for LS/HNPCC patients. However, none of the genetically defined MMR-deficient mouse lines reported to date develop colon tumors (30), unless combined with other defective alleles such as *Apc^{Min/+}*. The strength of the *Msh2^{flox/flox}VpC^{+/+}* model is that it restricts *Msh2* deletion to a biologically relevant tissue compartment, the intestinal epithelial crypt cells (24). The normal life span of both control groups also indicates that intestinal-specific expression of Cre alone has no discernible phenotype in this mouse background, unlike other systems (46). The *Msh2^{flox/flox}VpC^{+/+}* mice probably present the best available rodent system for preclinical modeling of LS/HNPCC. This model is amenable to further refinements which may align its resultant tumor phenotype more exactly with that arising in LS/HNPCC patients. Treatment with azoxymethane/dextran sodium sulphate (AOM/DSS) can be used to expand the tumor incidence to the colon (47). It is also possible to restrict *Msh2* deletion to only the colon using a recently reported surgical procedure to physically limit subsequent infection of a Cre expressing adenovirus (48).

Recent clinical trials report that regular aspirin use is associated with a lower risk of cancer-specific mortality in individuals already diagnosed with colorectal (49) or breast cancer (50). The prevailing consensus is that the chemopreventive benefits of aspirin only manifest after ten years or longer of continuous administration (3, 4). Our LS/HNPCC mouse data indicates that ASA and low dose NO-ASA requires continuous long-term treatment, with a subsequent 18–21% increase in lifespan. Considering that these animals are homozygous nulls for *Msh2* (in the intestine), whereas LS/HNPCC patients are heterozygous for MMR defects and require a “second-hit” to promote tumorigenesis, this increase could translate into a dramatic difference in age-of-onset. Indeed, recently disclosed data suggest that long-term ASA treatment of LS/HNPCC patients appears to result in a 50% decrease in tumor incidence (J. Burn, personal communication).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ASA and low dose NO-ASA promote survival of LS/HNPCC mice

Kaplan-Meier survival plots are shown for *VpC*^{+/+} and *Msh2*^{flox/flox}*VpC*^{+/+} mice. The median survival times for each group were as follows: *VpC*^{+/+}; untreated and 720 mg/kg ASA, >800 days; *Msh2*^{flox/flox}*VpC*^{+/+} untreated, 400 mg/kg ASA, 72 mg/kg NO-ASA, 720 mg/kg NO-ASA and 1500 mg/kg NO-ASA; 333, 393, 403, 229 and 175 days, respectively. Any pair-wise comparison between groups of *Msh2*^{flox/flox}*VpC*^{+/+} mice (log-rank test) was significant, with $P < 0.0001$, except untreated mice versus 400 mg/kg ASA ($P = 0.0007$) and untreated mice versus 72 mg/kg NO-ASA ($P = 0.0003$).

Figure 2. LS/HNPCC mice eventually develop intestinal tumors regardless of ASA or NO-ASA treatment

A, Representative panels of the gross pathologies that develop in the duodenal and proximal jejunal regions of LS/HNPCC mice. LS/HNPCC mice treated with 400 mg/kg ASA and 72 mg/kg NO-ASA survived, on average, 20% longer than untreated mice (393/403 versus 333 days) but did not develop greater tumor burdens. In contrast, NO-ASA treatments of 720 mg/kg and 1500 mg/kg resulted in the development of multiple tumors in these regions. Tumors/lesions are indicated by the black arrow heads. (See Table 1 also.) B, LS/HNPCC mice develop similar types of lesions, all epithelial, regardless of drug treatment: upper left panel, untreated, foci of epithelial hyperplasia; upper right panel, 720 mg/kg NO-ASA, adenoma; lower left panel, 400 mg/kg ASA, adenocarcinoma; lower right panel, 1500 mg/kg NO-ASA, adenocarcinoma. Scale bars: 300 μ m. (See Supplementary Table S1.)

Figure 3. Intestinal tissues of NO-ASA treated LS/HNPCC mice demonstrate persistent MSI

A–F, MSI was evaluated by analysis at five microsatellite markers, MSM01–05, for matched sets of three samples: ear (E), intestinal tumor (T) and adjacent normal (N) intestinal tissue. Data is only shown for four markers, as MSM02 was uninformative in all cases. The MSS ear tissues are representative of the standard allele sizes of these markers, indicated by 0 in each panel to signify 0 bp deviation from normal. Differences in the lengths of microsatellite markers between normal and tumor samples and their cognate ear samples, indicated in the top right corner of each panel, are expressed in units of 1 bp (mononucleotide) or 2 bp (dinucleotide). Vertical grey bars indicate the position of each major allele in the ear tissues. (See Supplementary Table S2).

Figure 4. Continual dietary exposure to ASA may partially stabilize the inherent MSI of intestinal tissues

A–F, Small-pool PCR was performed with the dinucleotide marker MSM01 on subsets of matched tissue samples (Ear, Normal, Tumor) from LS/HNPCC mice. The microsatellite profiles resulting from forty-eight individual reactions have been translated into allele distributions, shown by the horizontal grey bars, and the major allele size within each profile has been emphasized in color. In the example depicted by the middle histogram, the MSI profile shown has been translated onto the *x-axis* as a grey bar ranging from 127–139 bp,

highlighted with two major alleles of 133 bp and 135 bp. The major allele size of MSM01 (137 bp) in MSS ear tissue is indicated by ▲. Changes in microsatellite length occur in 2 bp increments: gold 141 bp; black 137 bp; red 135 bp; green 133 bp; blue 131 bp; plum 129 bp.

Table 1

Intestinal tumor burdens after dietary exposure to ASA and NO-ASA

treatment	n	n(T)	tumors	tumors per intestinal region				total	tumors / n mice	tumors / n(T) mice
				duodenum	jejunum	ileum	colon			
VpC+/+ 720 mg/kg NO-ASA	22	3	total	2	1	0	0	3	0.13 ± 0.34	1.00 ± 0.00
			mean	0.09 ± 0.29	0.04 ± 0.21	0	0			
Msh2flox/floxVpC+/+ plain food	24	20	total	11	26	1	0	38	1.58 ± 1.31	1.90 ± 1.02
			mean	0.46 ± 0.66	1.08 ± 1.06	0.04 ± 0.20	0			
Msh2flox/floxVpC+/+ 400 mg/kg ASA	26	22	total	8	27	2	0	37	1.42 ± 0.99	1.68 ± 0.72
			mean	0.31 ± 0.47	1.04 ± 0.87	0.08 ± 0.27	0			
Msh2flox/floxVpC+/+ 72 mg/kg NO-ASA	15	14	total	6	18	1	0	25	1.67 ± 1.34	1.78 ± 1.25
			mean	0.40 ± 0.63	1.20 ± 0.86	0.07 ± 0.26	0			
Msh2flox/floxVpC+/+ 720 mg/kg NO-ASA	32	31	total	67	89	2	0	158	// 4.94 ± 2.91	## 5.10 ± 2.81
			mean	* 2.09 ± 1.75	† 2.78 ± 1.81	0.06 ± 0.24	0			
Msh2flox/floxVpC+/+ 1500 mg/kg NO-ASA	25	25	total	58	66	1	0	125	** 5.00 ± 1.73	## 5.00 ± 1.73
			mean	† 2.32 ± 1.03	§ 2.64 ± 1.47	0.04 ± 0.20	0			

means are ± standard deviation

n= total number of mice per group; n(T)= total number of tumor bearing mice:

* P 0.0005;

† P <0.0001;

‡ P 0.0030;

§ P 0.0016;

// * P 0.0002;

** P <0.0001 (Mann-Whitney U test) when compared to Msh2flox/floxVpC+/+ mice groups receiving either plain food, 400 mg/kg ASA or 72 mg/kg NO-ASA.

P <0.0001 (Mann-Whitney U test) when compared to (tumors / n(T) mice) for Msh2flox/floxVpC+/+ mice groups receiving either plain food, 400 mg/kg ASA or 72 mg/kg NO-ASA.

Table 2

Comparative MSI analyses of intestinal tumors from LS/HNPCC mice

Treatment	n	MSS	Comparative MSI (N v T)			* Total MSI
			MSI-L	MSI-H	MSI	
VpC ^{+/+} 720 mg/kg NO-ASA	16	‡ n/a	‡ n/a	‡ n/a	‡ n/a	‡ n/a
Msh2 ^{flax/flax} VpC ^{+/+} plain food	18	5 (28)	7 (39)	6 (33)	13 (72)	
Msh2 ^{flax/flax} VpC ^{+/+} 400 mg/kg ASA	18	4 (22)	10 (56)	4 (22)	14 (78)	
Msh2 ^{flax/flax} VpC ^{+/+} 72 mg/kg NO-ASA	13	2 (15)	5 (39)	6 (46)	11 (85)	
Msh2 ^{flax/flax} VpC ^{+/+} 720 mg/kg NO-ASA	16	1 (6)	3 (19)	12 (75)	15 (94)	
Msh2 ^{flax/flax} VpC ^{+/+} 1500 mg/kg NO-ASA	14	2 (14)	7 (50)	5 (36)	12 (86)	

* Total MSI = (MSI-L + MSI-H)

‡ not applicable; all tissues were MSS