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# NO-donating aspirin inhibits intestinal carcinogenesis in *Min* ($APC^{Min/+}$) mice

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**Abstract**

The chemopreventive effect of nitric oxide-releasing aspirin (NO-ASA) against gastrointestinal tumorigenesis was evaluated in *Min* ($APC^{Min/+}$) mice. NO-ASA consists of a traditional ASA that bears covalently attached to it an NO-releasing moiety. Four groups ($N = 10$) of six-week-old female C57BL/6J $APC^{Min/+}$ and the corresponding C57BL/6J$^{+/+}$ wild type mice were treated either with vehicle or NO-ASA 100 mg/kg/day intrarectally for 21 days. There were no signs of overt toxicity including gastrointestinal toxicity from NO-ASA. Vehicle treated *Min* mice had $24.7 \pm 3.8$ tumors (mean $\pm$ SEM) and NO-ASA treated *Min* mice had $10.1 \pm 1.4$ tumors (59% reduction; $P < 0.001$). Wild type mice showed no tumors. NO-ASA did not affect cell proliferation in small intestinal mucosa, determined by immunohistochemical staining for PCNA. Our findings establish the strong inhibitory effect of NO-ASA in intestinal carcinogenesis in the *Min* mouse and suggest that this agent merits further evaluation as a chemopreventive agent against colon cancer.


*Keywords:* Nitric oxide-releasing aspirin; Aspirin; Nitric oxide; Colon cancer; Chemoprevention; Proliferation; *Min* mice

The observation that non-steroidal anti-inflammatory drugs (NSAIDs) prevent the development of colon and other cancers in humans has revolutionized our approach to colon cancer prevention [1]. Aspirin (ASA) has emerged as the prototypical chemoprevention agent against colon cancer. However, that NSAIDs, including ASA, prevent cancer in less than half of the subjects and are associated with significant side effects has provided the impetus to develop agents that are safer and more efficacious than NSAIDs [2–4]. Nitric oxide-releasing NSAIDs (NO-NSAIDs) represent such an approach [5]. They consist of a traditional NSAID that bears covalently attached to it an NO-releasing moiety. Compared to their traditional counterparts, NO-NSAIDs have superior potency in inhibiting the growth of colon and other cancer cell lines in vitro, with NO-ASA being the most potent among them [6]. A recent study in humans showed that the gastrointestinal toxicity of NO-ASA is equivalent to that of placebo [7]. In light of these considerations, we evaluated whether NO-ASA inhibits the development of gastrointestinal tumors in the *Min* ($APC^{Min/+}$) mouse model of intestinal cancer. *Min* mice have a truncating mutation in the *Apc* gene that predisposes them to the development of gastrointestinal tumors, mainly in the small intestine [8]. In many important ways, this model system recapitulates the salient steps of colon carcinogenesis and thus represents a useful (and extensively utilized) experimental system. Here we report our observations on the effect of NO-ASA on intestinal carcinogenesis in *Min* mice, showing a profound inhibitory effect.

## Materials and methods

*Reagents.* NO-ASA (*NCX4040*, 2-(acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester), a gift from NicOx, SA, Sophia Antipolis,

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France, was suspended (35 mg/ml, wt/v) in a solution of 0.5% carboxy methylcellulose (Sigma Chemical, St. Louis, MO).

*Animals and treatment protocol.* Six-week-old female C57BL/6J $APC^{Min/+}$ mice and the corresponding C57BL/6J$^{+/+}$ wild type mice (of which the *Min* mice are a congenic derivative) were purchased from Jackson Laboratories, Bar Harbor, ME. After acclimation, the animals were housed and maintained according to the approved standards of Institutional Animal Care and Use Committee. Mice were divided into groups of 10 each and treated via intrarectal administration as follows: *group 1*, wild type controls treated with vehicle; *group 2*, wild type controls treated with NO-ASA 100 mg/kg/day; *group 3*, *Min* mice treated with vehicle; and *group 4*, *Min* mice treated with NO-ASA 100 mg/kg/day. After 21 days of treatment, all animals were euthanized and their small intestine was dissected. Tumors were counted under a magnifying lens and tissue samples were preserved in formalin.

*Morphological evaluation and immunohistochemistry for PCNA expression.* Tissue samples were fixed in 10% formalin and embedded in paraffin, and 4 μm thick sections were placed on slides. The tissue sections were dewaxed and rehydrated according to standard protocols. For morphological analysis, tissue sections were stained with hematoxylin and eosin. A pathologist evaluated the tissue sections blinded to their identity. The expression of proliferating cell nuclear antigen (PCNA) was determined by immunohistochemical staining. We followed the methodology for rapid immunohistochemical staining using the Vectastain universal quick kit (Vector Laboratories, Burlingame, CA). Tissue sections were initially reacted with the anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 dilution), followed by the procedure of the Vectastain universal quick kit. As a negative control, for each sample, a successive tissue section was reacted with an isotypic nonspecific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Tissue sections were counterstained with hematoxylin, mounted with permount, and viewed by light microscopy. We selected for scoring only villi sectioned longitudinally through their entire length. Cells with a blue nucleus were considered unlabeled and cells with a brown nucleus were considered labeled. We scored separately the crypt cells and the epithelial cells lining the surface of the small intestine (villous surface). We calculated the proliferation index (PI) for each of the two segments separately. This was obtained by dividing the number of labeled cells by the total number of cells and multiplying by 100.

## Results and discussion

### *NO-ASA did not affect the body weight of mice—no evidence of gastrointestinal toxicity*

There was no statistically significant difference in the body weight of the four groups of mice at either the beginning or the end of the study (Table 1) nor during its course (data not shown). At necropsy, there was no evidence of gastric or other gastrointestinal toxicity such as mucosal hyperemia, erosions, and ulcers or bleeding.

### *NO-ASA reduces tumor multiplicity in Min mice*

The small intestine is the part of the gastrointestinal tract where by far the greatest number of tumors develop in this animal tumor model [8]. As shown in Figs. 1 and 2, tumor multiplicity (i.e., the number of tumors per animal) in the small intestine of *Min* mice was greatly reduced following treatment with NO-ASA. Vehicle treated *Min* mice had $24.7 \pm 3.8$ tumors (mean $\pm$ SEM) for this and all subsequent values) whereas, NO-ASA treated *Min* mice had $10.1 \pm 1.4$ tumors. This represents a 59% reduction in the number of tumors following treatment with NO-ASA and the difference between the two groups is statistically significant ($p < 0.001$, Student's $t$ test). As expected, no tumors were observed in any of the wild type animals. Although we did not measure the volume of each tumor, the tumors seen in *Min* mice treated with NO-ASA were visibly smaller than those observed in *Min* mice treated with vehicle alone. Tumor incidence was not affected by NO-ASA, as only one of the NO-ASA treated *Min* mice had no detectable intestinal tumors.

### *Effect on epithelial cell proliferation*

Proliferation is an important cell kinetic parameter that determines, often in a critical way, tumor mass [9]. Since NO-ASA inhibits cell proliferation in several cancer cell lines [6,10], we assessed cell proliferation in the small intestine of *Min* and control mice in response to NO-ASA treatment (Fig. 3). As shown in Table 1, NO-ASA treatment had no effect on cell proliferation in either wild type or *Min* mice.

Our results document that NO-ASA exerts a profound inhibitory effect on colonic carcinogenesis in *Min* mice without any overt signs of toxicity. These results are the first report documenting the tumor inhibitory

Table 1
The effect of NO-ASA on *Min* and wild type mice

| Group ($N$) | Body weight (g) | | Proliferation index | |
|---|---|---|---|---|
| | Start | End | Crypt | Villous surface |
| | mean $\pm$ SEM | | | |
| Wild type | | | | |
| Vehicle (10) | $16.8 \pm 0.4$ | $18.3 \pm 0.3$ | $97.3 \pm 1.0$ | $12.9 \pm 2.0$ |
| NO-ASA (10) | $16.9 \pm 0.3$ | $18.7 \pm 1.8$ | $98.2 \pm 1.1$ | $9.8 \pm 1.8$ |
| *Min* | | | | |
| Vehicle (10) | $16.5 \pm 0.4$ | $18.3 \pm 0.2$ | $98.9 \pm 1.2$ | $8.9 \pm 0.8$ |
| NO-ASA (10) | $16.4 \pm 0.5$ | $18.1 \pm 0.5$ | $99.1 \pm 0.9$ | $12.3 \pm 0.4$ |

Stats: differences are not statistically significant.

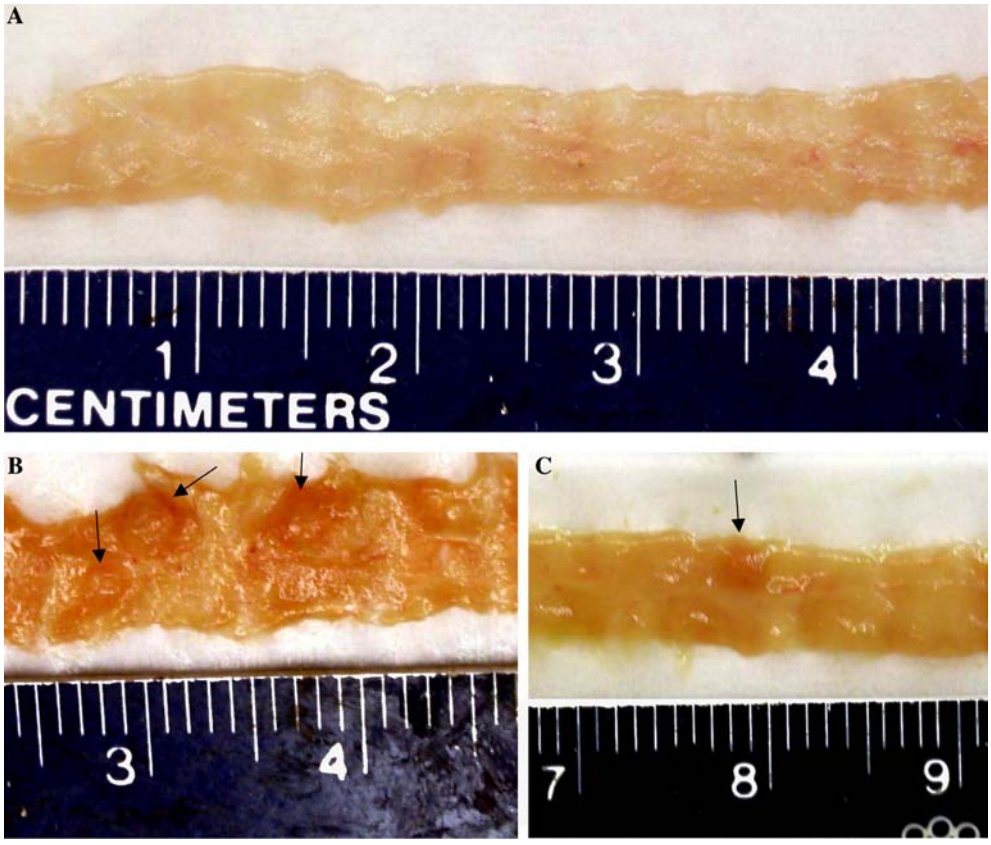


Fig. 1. Dissected small intestines. (A), wild type control showing no tumors; (B), $APC^{Min/+}$ mice showing the presence of tumors (arrows); (C), $APC^{Min/+}$ mice treated with NO-aspirin showing the reduced number of tumors.

effect of NO-ASA in an animal model of intestinal cancer. Previous reports using aberrant crypt foci as an early biomarker of colon carcinogenesis have generated similar results [11,12], although both studies used different NO-ASA compounds (different spacer linking ASA to the NO-releasing moiety or different positional isomers).

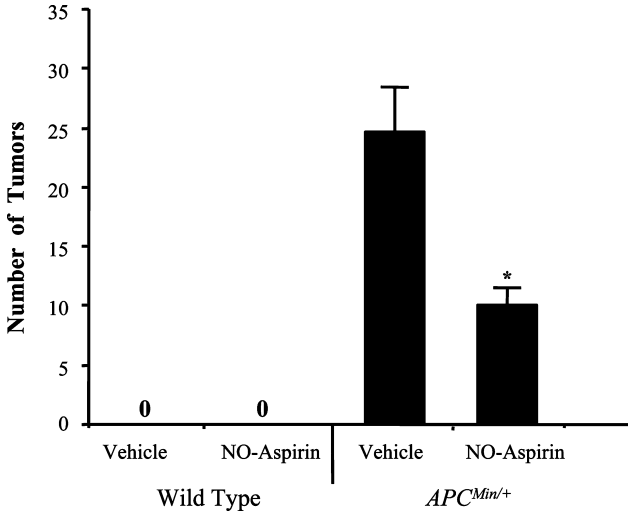


Fig. 2. Effect of NO-ASA on tumor multiplicity in *Min* mice. The number of tumors per animal was counted in various treatment groups as indicated above. The results show that the number to tumors in the small intestine of *Min* mice was greatly reduced following treatment with NO-ASA (*$P < 0.001$, Student's $t$ test).

There are three points that merit special emphasis. First, the effect of NO-ASA is quantitatively very significant, even though animals were treated for a brief period of time. For example, in one of the reported studies, *Min* mice were treated with a COX-2 inhibitor more than twice as long (for over 7 weeks) to achieve comparable results [13]. One can venture the prediction that, had these animals been treated for a longer period of time or had their treatment started earlier, the effect of NO-ASA would have been even more pronounced. Second, the route of administration of NO-ASA (intrarectal instillation) differs from that employed in almost all published reports; in chemoprevention studies NSAIDs are often administered either through the drinking water or admixed with their food. The route of administration may affect the pharmacological effect of NO-ASA as previously demonstrated for other chemopreventive agents [14]. In our case, intrarectal administration has likely bypassed, at least partially, initial liver metabolism of this compound ("first-pass effect"). Third, NO-ASA had no effect on PCNA expression. This finding underscores the complexity of the mechanism by which this versatile compound exerts its

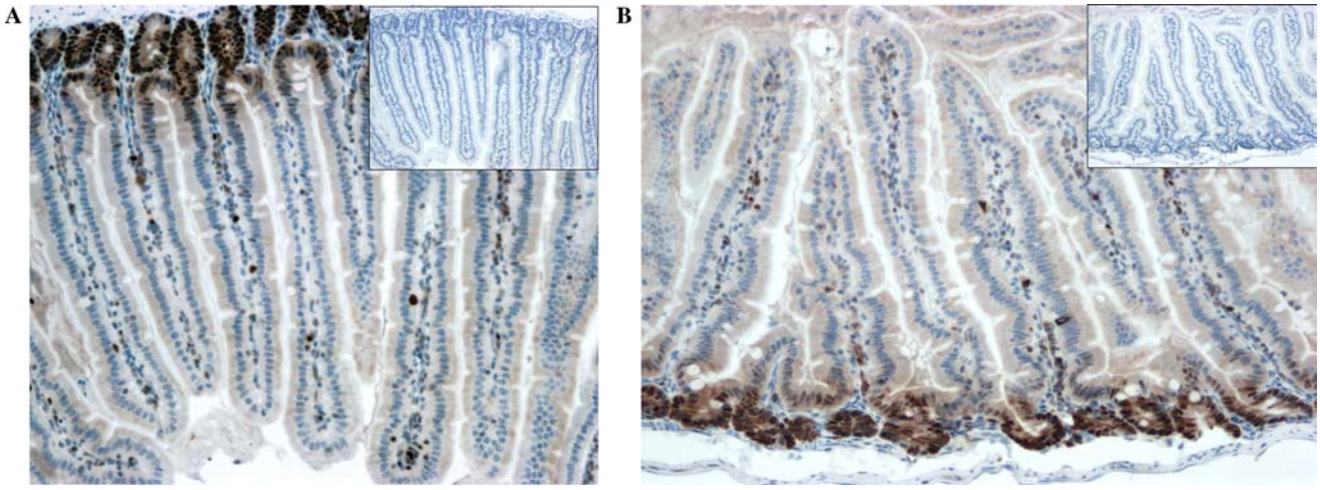


Fig. 3. PCNA staining of the small intestinal mucosa of NO-ASA treated and control *Min* mice. Normal-appearing small intestinal mucosa from *Min* mice treated with vehicle (A) or NO-ASA (B), as in Materials and methods. There is no difference in PCNA expression between them. Insets are successive tissue sections treated with a non-specific isotypic control antibody. Magnification 200×.

chemopreventive activity. In fact, based on in vitro data, we have speculated elsewhere that cell killing (not restricted to apoptosis) may be the primary mode of action of NO-ASA [10].

Although our current data do not provide any clues to the molecular mechanism by which NO-ASA acts in preventing intestinal carcinogenesis, it is likely that this effect represents the sum total of several of its pleiotropic effects on cancer cells. Prominent among them is the inhibitory effect of NO-ASA on TCF/β-catenin signaling [15]. This pathway is considered a critical determinant of the fate of the colonocyte, and thus its suppression by pharmacological agents such as NO-ASA can impact carcinogenesis in a major way. In addition, NO-ASA can affect other pathways, such as NF-κB and nitric oxide synthase 2, and this mechanistic redundancy may be an important feature of its mechanism of action against cancer [16]. Regardless of mechanistic issues, the inhibitory effect of NO-ASA on intestinal tumorigenesis in *Min* mice is in excellent agreement with our previous in vitro work that indicated the chemopreventive potential of this novel class of compounds. In vitro, NO-ASA is 2500–5000-fold more potent than traditional ASA in inhibiting the growth of colon cancer cells [6].

In conclusion, our findings establish the strong inhibitory effect of NO-ASA in intestinal carcinogenesis in the *Min* mouse and the absence of any overt toxicity and suggest that this agent merits further evaluation as a potentially effective and safe chemopreventive agent against colon cancer.

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