# The use of nitric oxide-donating nonsteroidal anti-inflammatory drugs in the chemoprevention of colorectal neoplasia

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**Purpose of review**

Nitric oxide-donating nonsteroidal anti-inflammatory drugs are emerging as a promising class of compounds for the chemoprevention of colon cancer. Recent progress in their preclinical and mechanistic evaluation is reviewed.

**Recent findings**

Compared to their parent compounds, nitric oxide-donating nonsteroidal anti-inflammatory drugs are up to several hundred times more potent in inhibiting the growth of colon cancer cell lines and also quite effective in preventing colon cancer in various tumor animal models. Their chemopreventive effect is brought about through a strong cell kinetic effect, including inhibition of proliferation, induction of cell death and inhibition of cell cycle phase transitions. The induction of oxidative stress appears mechanistically crucial. Pleiotropic effects on cell signaling have been identified including Wnt, NOS2, mitogen-activated protein kinase and Nrf2 signaling. Nitric oxide-donating nonsteroidal anti-inflammatory drugs, particularly nitric oxide-donating aspirin, appear to be very safe compounds, as evidenced from many animal and early human studies.

**Summary**

Nitric oxide-donating nonsteroidal anti-inflammatory drugs hold the promise of being safe and effective chemopreventive agents against colon cancer. Clinical trials are needed to determine whether these drugs can be applied clinically.

**Keywords**

cancer prevention, colon cancer, nitric oxide, nitric oxide-donating aspirin, nitric oxide-donating nonsteroidal anti-inflammatory drugs

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**Abbreviations**

| | |
|---|---|
| **ASA** | aspirin |
| **COX** | cyclooxygenase |
| **NO** | nitrc oxide (donating) |
| **NSAID** | nonsteroidal anti-inflammatory drug |


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## Introduction

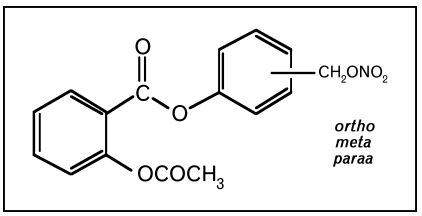
Chemoprevention, defined as the application of natural or synthetic agents to prevent the development or recurrence of cancer, is a highly promising approach to control colon cancer. Extensive epidemiological data and interventional studies have established that conventional nonsteroidal anti-inflammatory drugs (NSAIDs) prevent colon cancer, but with less than 50% efficacy. The limited efficacy and untoward effects that accompany NSAIDs have prompted the search for better performing agents. Nitric oxide-donating (NO)-NSAIDs, conceived as a safe alternative to conventional NSAIDs, and initially intended for rheumatologic and cardiovascular applications [1], are now under intense study for their anticancer properties.

## Structure and metabolism of nitric oxide-donating nonsteroidal anti-inflammatory drugs

The defining feature of NO-NSAIDs is their ability to release NO, one of the smallest yet most powerful biological molecules. Figure 1 shows the structure of NO-aspirin (NO-ASA), at present the best-studied NO-NSAID. The three structural components of NO-NSAIDs, conceptually the same for all, are the conventional NSAID (aspirin in this case), the NO-donating moiety ($-CH_2ONO_2$) and the spacer that links the two. The spacer can vary in its chemical structure, providing a great number of NO-NSAIDs. Given the ease of formation of these nitrate-ester compounds, for a given spacer one could predict a number of derivatives almost equal to the number of available conventional NSAIDs. In the case shown here, the benzene ring of the spacer provides three positional isomers (*ortho*, *meta* and *para* with respect to the ester bond linking ASA and spacer), further increasing the range of NO-donating molecules.

NO-NSAIDs are part of a larger family of NO-related therapeutic agents that include prodrugs that elevate NO levels, scavengers of NO and inhibitors of NO synthesis

**Figure 1 The chemical structure of nitric oxide-donating aspirin**




Conventional aspirin (acetylsalicylic acid) is covalently bound through an aromatic spacer molecule to the nitric oxide-donating moiety ($-CH_2ONO_2$).

[2]. For example, Thatcher's group [2] has developed NO-chimeras (a pharmacophore plus an NO mimetic group) and Velazquez *et al.* [3] synthesized diphenyl-oxadiazoles as cyclooxygenase (COX)-2 inhibitor/NO donor agents, expecting low ulcerogenicity and no cardiovascular adverse effects. The role in cancer prevention of such novel compounds, although plausible, is at present uncertain.

A key question concerning NO-NSAIDs has been whether (and by which mechanism) they release NO. Recently, Govoni *et al.* [4•], using electron paramagnetic resonance, provided direct evidence that NO-flurbiprofen generated NO in erythrocytes, with hemoglobin mediating this biotransformation. Their work and that of others suggests that the NO-releasing moiety ($-ONO_2$) undergoes one-electron reduction to $NO_2^-$, which is then either converted to NO or oxidized to $NO_3^-$. They also demonstrated that NO-flurbiprofen released NO at a slower rate than nitroglycerin, and this may account at least in part for the NO-NSAIDs' lack of a hypotensive effect (in contrast to organic nitrates).

To date three groups have explored the metabolism of NO-NSAIDs [4•,5,6]. The general pattern that emerges is that these compounds first undergo a rapid hydrolysis of the carboxyl ester, thus releasing the parent NSAID and the spacer moiety bearing the NO-releasing group attached to it. This step is likely catalyzed by esterases, which have not yet been identified. The second and slower step involves the release of NO, which may proceed as described above. Cytosolic glutathione-*S*-transferases in the liver are involved in these biotransformations. Of interest, neither intact NO-NSAIDs nor their denitrated derivatives have been detected in the circulation. Contrary to initial assumptions, following its oral administration NO-ASA (the only one studied) survives essentially intact in the stomach, with its *O*-deacetylated derivative being the only metabolite there at very low concentrations [5]. Whether this accounts for the apparent safety of NO-ASA remains unclear.

## Chemopreventive effects against colon cancer

The first evidence that NO-NSAIDs possess chemopreventive properties was provided by Williams *et al.* [7], who showed that NO-ASA, NO-sulindac and NO-ibuprofen reduced the growth of cultured HT-29 human colon adenocarcinoma cells much more potently than their corresponding NSAIDs. Subsequent studies showed that this is true of several NO-NSAIDs whose potency can be enhanced by up to several hundred times compared to their parent compounds [8]. Their growth-inhibitory effect was due to inhibition of proliferation, induction of apoptosis and also inhibition of cell cycle phase transitions. These observations were later expanded to document two types of cell death induced by NO-ASA – classical apoptosis and a form of cell necrosis termed atypical cell death.

Gao *et al.* [9] mapped in detail the induction of apoptosis by NO-ASA in the SW480 colon cancer cell line. The first and crucial step was the induction of oxidative stress evidenced by enhanced levels of reactive oxygen species. Reduced levels of glutathione, the major antioxidant in mammalian cells, by NO-ASA accounted for part of this initiating event. Subsequent steps included activation of the intrinsic apoptosis pathway and also inhibition of Wnt signaling. The latter had two components: blocking the formation of the transcriptionally active complex between β-catenin and T cell factor, and cleaving β-catenin at high NO-ASA concentrations. The atypical cell death, initially described *in vitro*, may actually occur *in vivo*. Ouyang *et al.* [10] recently captured a sequence of morphological changes in intestinal tumors of *Min* mice treated with NO-ASA that strongly suggest its existence. The importance of the induction of apoptosis for the chemopreventive effect of NO-ASA was underscored by the finding that the apoptosis index in the intestine paralleled the chemopreventive effect of NO-ASA isomers: *para* NO-ASA reduced intestinal tumor incidence by 59% and *meta* NO-ASA by 38%, whereas apoptosis for *para* was 52.5% for normal mucosa and 70.3% for tumors, and for *meta* was 31% and 22%, respectively [10]. Potentially interesting is the observation that NO-ASA induced apoptosis in *Min* mice, but did not do so in their wild-type congenic controls.

Recently published animal studies have confirmed and expanded the original observation of Bak *et al.* [11] that NO-ASA inhibits colon carcinogenesis more effectively than conventional ASA. Congruent results have been obtained from *Min* mice, a veritable genetic model of intestinal carcinogenesis, rats treated with the carcinogen azoxymethane and tumor xenografts. The *Min* mice data were summarized above. The rat study, which followed a classical chemoprevention protocol and had as an end-point the formation of carcinoma, evaluated

NO-indomethacin and *meta* NO-ASA [12]. NO-ASA 3000 p.p.m. reduced tumor incidence by 48% and tumor multiplicity by 73%, whereas the corresponding values for NO-indomethacin 80 p.p.m. were 69 and 76%. During the 1 year of their administration these drugs caused no toxicity in the gastrointestinal tract or any of several organs that were examined. Finally, *para* NO-ASA reduced the weight of colon cancer cell line xenografts by 40% [13].

What emerges from these and the remainder of animal studies reported to date is that, despite individual variations, NO-NSAIDs effectively inhibit colon carcinogenesis – their chemopreventive effect reaching as high as 76% [12]. Moreover, whenever a direct comparison to conventional ASA was performed, NO-ASA was more effective (e.g. [11]).

## Mechanism of action of nitric oxide-donating nonsteroidal anti-inflammatory drugs

The mechanism of action of NO-NSAIDs against colon cancer has been pursued from several angles: their cell kinetic effect (outlined above), structure–activity relationships, effects on cell signaling pathways and effects on detoxifying enzymes.

Structure–activity studies have assessed the role of the NO-releasing moiety, and, for NO-ASA, the role of positional isomerism and of the acetyl group, which accounts for ASA's best-known pharmacological activity, i.e. the inhibition of COX. The NO-donating moiety appears indispensable for the ability of NO-NSAIDs to inhibit cancer cell growth. Direct support for this notion came from two sets of results: (1) the rate of release of NO by the three positional isomers of NO-ASA and one with an aliphatic spacer was directly correlated with their potency to inhibit cell growth, and (2) denitrated analogs of NO-ASA failed to have a significant effect on cell growth or other parameters that were examined [9,13,14]. A rather surprising finding, not yet fully appreciated or explained, was the striking effect of positional isomerism of NO-ASA on its pharmacological properties. The *ortho* and *para* isomers showed similar $IC_{50}$ values (1–5 μmol/l) for colon cancer cell growth inhibition, whereas the $IC_{50}$ of the *meta* isomer was 200–500 μmol/l. An animal study showed a similar, but less pronounced difference in efficacy [14]. That deacetylated analogs of NO-ASA had indistinguishable effects on colon cancer cell growth compared to the full molecule suggests that COX acetylation (i.e. inactivation) by NO-ASA is not required, at least for its in-vitro effect [14].

Several signaling pathways have been assessed in terms of their response to NO-NSAIDs, mainly NO-ASA. A recent study examined the effect of NO-ASA on mitogen-activated protein kinase signaling in HT-29 colon cancer cells [15]. NO-ASA had its greatest effect on the JNK and p38 pathways, whose activation was accompanied by large increases in the phosphorylation of the downstream transcription factors c-Jun and ATF-2. With the aid of mitogen-activated protein kinase inhibitors and small interfering RNA gene silencing, it was demonstrated that NO-ASA blocks cell growth only when it inhibits both p38 and JNK. Using the same cell line, another recent study showed that NO-ASA inhibited both the expression and the catalytic activity of the inducible form of NO synthase – an enzyme implicated in colon carcinogenesis [16]. Such inhibition was noted in colonic tumors as well [12].

COX-2 has been considered a key molecule in colon carcinogenesis and the application of COX-2 inhibitors to colon cancer prevention has been pursued vigorously. In addition, COX inhibition by ASA has been used to prevent cardiovascular events. Thus, it is not surprising that the effect of NO-ASA on COX has been examined closely. This is, however, an area of some uncertainty. Williams *et al.* [17] first reported that NO-ASA induces the expression of COX-2 in cell lines and also that inhibition of COX-2 is not required for the effect of NO-NSAIDs on colon cancer cell growth. Tesei *et al.* [13], however, failed to observe induction of COX-2 by NO-ASA in two cell lines different from those used by Williams *et al.*. Corazzi *et al.* [18], studying human blood, recently reported that NO-ASA inhibits the activity of both COX-1 and COX-2. This inhibition is mainly due to the acetyl group of the ASA moiety of NO-ASA, with NO making at best a modest contribution. An animal study also showed that both NO-ASA and NO-indomethacin inhibited COX-1 and COX-2 activity in the colonic mucosa of azoxymethane-treated rats [12].

Finally, a recent study examined whether NO-ASA modulates drug-metabolizing enzymes; such modulation leading to facilitated elimination of carcinogens is considered a successful strategy for cancer chemoprevention [19]. In colon and liver cell lines, and also in the liver and intestine of *Min* mice, NO-ASA induced the activity and expression of NAD(P)H:quinone oxireductase and glutathione-*S*-transferase. In contrast to phase II enzymes, NO-ASA had only a marginal effect on P450 1A1 and P450 2E1 (two phase I enzymes). Paralleling the induction of NAD(P)H:quinone oxireductase-1 and glutathione-*S*-transferase P1-1, NO-ASA induced the translocation of Nrf2 into the nucleus, likely by binding to Keap1, the protein that anchors Nrf2 in the cytoplasm.

These studies make it clear that at least NO-ASA modulates a large array of molecular targets. Each of these effects can in theory contribute to the chemopreventive effect of NO-ASA. It is unclear, however, whether only one, more than one or all of these effects are required for

the drug's overall pharmacological effect. Resolving this dilemma, formulated as mechanistic dominance (one effect) versus redundancy (multiple effects), will require further work [8]. The latter is, however, favored by us based on the complexity of the neoplastic phenotype and on the development, as a tumor grows, of subclones with different biochemical profiles. In other words, it may be precisely this pleiotropism that makes NO-ASA so effective.

## Safety of nitric oxide-donating nonsteroidal anti-inflammatory drugs

Safety is a most important aspect of any chemopreventive agent, including NO-NSAIDs. Extensive animal data, including those reviewed here, indicate that NO-NSAIDs have very limited side-effects, with particularly superior gastrointestinal safety. Human studies indicate the same. Healthy volunteers receiving NO-ASA for 1 week had no appreciable gastroduodenal damage, in contrast to those receiving conventional ASA who showed the expected endoscopic mucosal damage [20]. In a recent pilot study, pancreatic cancer patients receiving NO-ASA 1 g orally t.i.d. for 4 weeks for pain control experienced minimal side-effects [21]. Another study examined whether long-term use of NO-NSAIDs could be carcinogenic, given that NO can both promote and inhibit cancer. Since NO-NSAIDs are not yet in clinical use, Muscat *et al.* [22] examined subjects who had taken nitrates for many years for cardiological indications; NO release is nitrates' main mode of action. Analysis of the database of the Framingham Study revealed that long-term use of nitrates did not increase the incidence of colon cancer, whereas conventional ASA (internal control) produced in this cohort of patients the expected protective effect.

These encouraging early results, requiring confirmation by large-scale trials, suggest a most desirable feature for chemopreventive agents that would be administered long-term. What makes NO-NSAIDs so safe, if this is indeed proven, remains puzzling, especially since the parent compounds, which are released, can have by themselves significant toxicity.

## Conclusion

There is a clear and pressing need to develop safe and effective chemopreventive agents against colon cancer – the third most common cancer in the US. This need is even more compelling in view of the apparent limitations of conventional NSAIDs and COX-2-specific inhibitors. Ongoing evaluation of NO-NSAIDs attempts to address this need in the field of chemoprevention. A growing body of work, some of it reviewed here, provides internally consistent evidence that NO-NSAIDs may meet the two cardinal requirements of any candidate chemopreventive agent, i.e. efficacy and safety. Although much remains to be explored and explained, a rudimentary understanding of the underlying mechanism has already been achieved. At the present time, the studies that will in a sense decide the fate of these compounds regarding their application to chemoprevention are clinical trials that will ascertain their efficacy and safety after prolonged administration. An ongoing National Cancer Institute-sponsored clinical trial of NO-ASA for colon cancer prevention (conducted by the author) should provide an early assessment of NO-ASA. Mechanistic studies by an expanding number of laboratories around the world should generate insights that will lead to improved clinical applications of this promising class of compounds. The tantalizing possibility that NO-NSAIDs may play a part in colon cancer prevention appears now stronger than ever.

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 104).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