0022-3565/05/3123-978–988$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 312:978–988, 2005

Vol. 312, No. 3
75994/1192198
Printed in U.S.A.

Positional Isomerism Markedly Affects the Growth Inhibition of Colon Cancer Cells by Nitric Oxide-Donating Aspirin in Vitro and in Vivo

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Received August 12, 2004; accepted November 2, 2004

ABSTRACT

NO-donating aspirin (NO-ASA), a novel pharmacological agent currently undergoing clinical testing, consists of ASA to which a nitrate group is covalently linked via a spacer molecule. We synthesized the three positional isomers of NO-ASA with respect to the $-CH_2ONO_2$ group (*ortho*, *meta*, and *para*) and examined whether this isomerism affects the biological activity of NO-ASA on HT-29 human colon cancer cells. The *ortho*- and *para*-isomers showed similar IC_{50} values (1–5 μM) for cell growth inhibition over 72 h, whereas the IC_{50} of the *meta*-isomer was 200 to 500 μM. The *ortho*- and *para*-isomers inhibited cell proliferation more potently than the *meta*-isomer. All three induced apoptosis but the *ortho*- and *para*-isomers also induced atypical cells (they maintain their shape but have diminished or absent nuclear material). Treatment for 3 weeks of *Min* ($Apc^{min/+}$) mice, a model of intestinal cancer, with equimolar amounts of *meta*- and *para*-NO-ASA decreased the number of tumors in the small intestine by 36 and 59% ($P < 0.01$), respectively, compared with vehicle-treated controls, thus confirming their in vitro differences in potency. A structure-activity study of the three isomers revealed that substituting an aliphatic for the aromatic spacer or removing the $-ONO_2$ group profoundly diminished NO-ASA's ability to inhibit cell growth, whereas removal of the acetyl group on the ASA moiety did not affect cell growth inhibition. Thus, positional isomerism is critical for the pharmacological properties of NO-ASA against colon cancer and it should be taken into consideration in rational drug design.

Aspirin (ASA), the oldest of the nonsteroidal anti-inflammatory drugs (NSAIDs), is the prototypical drug for the prevention of colon cancer (Shiff and Rigas, 1999; Baron et al., 2003; Sandler et al., 2003). Despite initial enthusiasm, it appears that ASA is an unlikely candidate as a large-scale chemopreventive agent. There are two reasons that may preclude such an application: its limited efficacy, documented in recent clinical trials (Baron et al., 2003; Sandler et al., 2003) and its toxicity, mostly gastrointestinal and renal (Laine, 2003). Because of these limitations, attempts have been made to either formulate or synthesize a "safer and more effective aspirin". A promising approach in this direction has been the synthesis of NO-releasing aspirin (NO-ASA), a molecule designed to combine the pharmacological properties of traditional ASA with those of NO, a pivotal molecule not only for cardiovascular function but also for gastrointestinal mucosal physiology (reviewed in Rigas and Kashfi, 2004). Early results, including our own, indicate that NO-ASA displays, in general, superior efficacy and greater safety compared with traditional ASA (Fiorucci et al., 2003; Rigas and Kashfi, 2004). NO-ASA has a remarkable inhibitory effect on colon cancer cell growth, its IC_{50} being between 2500- and 5000-fold lower than that of ASA (Lavagna et al., 2001; Williams et al., 2001).

NO-ASA is derived from ASA by covalently attaching to it $-ONO_2$ through a chemical spacer; this modification is part of a general approach to nitrate pharmacologically useful compounds, including several NSAIDs. The structure of the NO-ASA molecule lends itself to the synthesis of several additional derivatives. We synthesized the three positional

This work was supported by National Institutes of Health Grants CA92423 and CA92423-S1.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.104.075994.

ABBREVIATIONS: ASA, aspirin; NSAIDs, nonsteroidal anti-inflammatory drugs; NO, nitric oxide; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; DETA NONOate, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PCNA, proliferating cell nuclear antigen; DAPI, 4,6-diamidino-2-phenylindole; PI, propidium iodide; COX, cyclooxygenase.

isomers of NO-ASA with respect to the $-ONO_2$ group and examined the effect of this isomerism on the biological activity of NO-ASA on colon cancer cells. To gain a better understanding of the structure-activity relationships of these isomers, we also synthesized a series of analogs for each one of them modifying groups considered or suspected to impact the biological activity of NO-ASA. There are at least two important reasons for studying these structure-activity relationships. First, this study can provide significant insights into the mechanism by which NO-ASA is so much more potent than its parent molecule. Second, the greatest possible pharmacological efficacy of NO-ASA is needed to optimize its envisioned clinical use. The ideal NO-ASA molecule should combine high efficacy at low doses that would provide even greater safety and maintain or enhance the wide spectrum of clinically useful effects of ASA, such as, for example, those on the heart (Sleight, 2003), the brain (Kirshner, 2003), and the colon.

In this report, we present the results of our study, which demonstrate that positional isomerism of NO-ASA can greatly influence its inhibitory effect on the growth of colon cancer cells. In addition, the structure-activity studies indicate that the $-ONO_2$ group of the molecule is very important with respect to its biological effects on colon cancer.

Materials and Methods

Reagents. Three NO-ASA isomers are 2-(acetyloxy)-2-[(nitrooxy)methyl]phenyl ester (NCX 4060, *ortho*-isomer), 2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (NCX 4016, *meta*-isomer), and 2-(acetyloxy)-4-[(nitrooxy)methyl]phenyl ester (NCX 4040, *para*-isomer); their deacetylated and denitrated analogs were synthesized by one of us (F.B.) according to the method of Penning et al. (1997). *S*-Nitroso-*N*-acetyl-penicillamine (SNAP), DETA NONOate, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), and human hemoglobin were from Sigma-Aldrich (St. Louis, MO). Other reagents were as described (following report).

Cell Lines. HT-29 human colon adenocarcinoma cells (American Type Culture Collection, Rockville, MD) were grown and treated as previously described (Williams et al., 2001). Cells were counted using a hemacytometer. Viability was determined by the trypan blue dye exclusion method.

Cell Proliferation Analysis. Cells (0.5×10^6) were fixed in 100% methanol for 10 min at −20°C, pelleted (5000 rpm × 10 min at 4°C), resuspended, and incubated in PBS containing 1% FBS/0.5% NP-40 on ice for 5 min. Cells were washed twice in PBS/1% FBS, pelleted, and resuspended in 50 μl of a 1:10 dilution of the anti-PCNA (proliferating cell nuclear antigen) primary antibody (PC-10; all antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS/1% FBS for 60 min at room temperature. Nonspecific IgG1/IgG2 was used as an isotypic control. Cells were then washed and incubated with goat-anti-mouse-phycoerythrin antibody (diluted 1:50) for 60 min at room temperature in the dark. Cells were washed again in PBS, resuspended in 500 μl of PBS/1% FBS containing 40 μg/ml propidium iodide (used to stain for DNA) and 200 μg/ml RNase type IIA, and analyzed within 30 min by flow cytometry using a Coulter Profile XL equipped with a single argon ion laser. For each subset, we analyzed >10,000 events. All parameters were collected in list mode files. Data were analyzed on an XL Elite Workstation (Beckman Coulter, Inc., Fullerton, CA) using the software program Multigraph.

Assays for Cell Death. The induction of apoptosis was determined by fluorescence microscopy of cells stained with 4,6-diamidino-2-phenylindole (DAPI; Accurate Chemical & Scientific, Westbury, NY). For each sample, at least five fields were examined. The morphological criteria used to identify apoptosis included cytoplasmic and nuclear shrinkage, chromatin condensation, and cytoplasmic blebbing with maintenance of the integrity of the cell membrane. As mentioned under *Results*, we also noted a distinct subpopulation of cells termed provisionally *atypical*. They exhibit diminished or no detectable DNA although they maintain the basic cellular structure (Williams et al., 2001). These cells are morphologically different from classical apoptotic cells. We also used the *annexin V assay* performed according to the manufacturer's instructions for "Rapid annexin V binding" (Oncogene Research Products, San Diego, CA). Briefly, both floating and trypsinized cells were washed with ice-cold PBS, followed by addition of the media binding reagent annexin V-FTIC, and following a 15-min incubation, addition of propidium iodide. Cells were analyzed immediately by flow cytometry as above.

NO Assay. NO levels in culture media were assayed using the Griess method that converts nitrate into nitrite. We used a commercially available kit (Cayman Chemical, Ann Arbor, MI) and followed the manufacturer's instructions.

Animal Study. 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 The Jackson Laboratory, Bar Harbor, ME. After acclimation, the animals were housed and maintained according to the approved standards of the 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 100 mg/kg/day *meta*-NO-ASA; group 3, wild-type controls treated with 100 mg/kg/day *para*-NO-ASA; group 4, *Min* mice treated with vehicle; group 5, *Min* mice treated with 100 mg/kg/day *meta*-NO-ASA; and group 6, *Min* mice treated with 100 mg/kg/day *para*-NO-ASA. NO-ASA (*meta*- or *para*-isomer) was administered suspended (35 mg/ml, w/v) in a solution of 0.5% carboxy methylcellulose (Sigma-Aldrich). After 21 days of treatment, all animals were euthanized, and their small intestine was dissected. Tumors were counted under a magnifying lens.

Statistical Analyses. Data are presented as mean ± S.E.M. for different sets of plates and treatment groups as indicated and compared using Student's *t* test.

Results

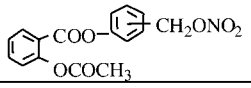
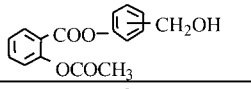
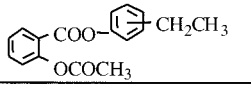
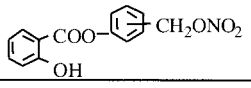
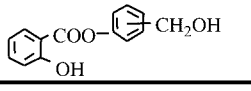
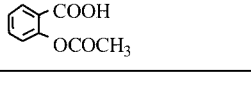
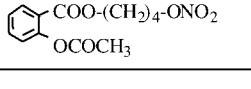
The Effect of NO-ASA Positional Isomers on Colon Cancer Cell Growth. HT-29 colon cancer cells, seeded at a density of 2.5×10^4 cells/cm^2, were exposed to the NO-ASA isomers and harvested every 24 h for 72 h. IC_{50} values were calculated from their growth curves (Table 1). The *ortho*- and *para*-isomers show similar IC_{50} values (1–5 μM), whereas the corresponding values for *meta*-isomer range between 200 and 500 μM, at least 100-fold higher than those of the other two. Compared with traditional ASA, all three isomers of NO-ASA were much more potent in reducing the growth of HT-29 colon cancer cells. The ratio of ASA/NO-ASA IC_{50} values at 48 and 72 h for the *meta*-isomer ranged between 8 and 13.5, whereas for the *ortho*- and *para*-isomers, it ranged between 937 and 2700. The 24-h values of these ratios can only be estimated as we were unable to obtain a precise IC_{50} value for ASA at 24 h. Nevertheless, it is likely that the 24-h ratios are within the ranges obtained for the other two time points. Thus, all three isomers are much more potent than ASA, whereas the *ortho*- and *para*-isomers are significantly more potent than the *meta*-isomer.

The Effect of NO-ASA Positional Isomers on Colon Cancer Cell Proliferation. To elucidate the mechanism underlying the effect of NO-ASA isomers on cell growth, we determined their effect on cell proliferation and cell death.

TABLE 1

IC_{50} (μM) values for structural analogues of NO-ASA in HT-29 cells

Cells were treated with various concentrations of the test agents shown above as described under *Materials and Methods*. Cell numbers were determined at 24, 48, and 72 h, from which IC_{50} values were calculated. Results are mean ± S.E.M. of three to five different experiments done in duplicate.

Structure	Compound	*Ortho*			*Meta*			*Para*		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
	Parent	2.8 ± 0.8	1.4 ± 0.3	1.0 ± 0.2	500 ± 62	370 ± 25	200 ± 15	5.0 ± 1	3.2 ± 2	2.2 ± 1
	Denitrated	> 100	> 100	> 100	> 1000	> 1000	> 1000	> 500	142 ± 15	155 ± 10
	Denitrated	> 200	115 ± 17	50 ± 10	> 1000	> 1000	> 1000	195 ± 11	54 ± 7	53 ± 9
	Deacetylated	6.5 ± 0.9	1.2 ± 0.2	0.8 ± 0.2	405 ± 37	418 ± 22	173 ± 17	2.5 ± 0.7	2.6 ± 0.6	2.2 ± 0.8
	Denitrated and Deacetylated	> 200	> 200	> 200	> 1000	> 1000	> 1000	684 ± 55	525 ± 18	316 ± 25
		24 hrs	48 hrs	72 hrs						
	Aspirin	> 5000	3000 ± 500	2700 ± 325						
	Aliphatic spacer	905 ± 42	460 ± 35	430 ± 42						

The former was assessed by determining the expression of PCNA in HT-29 cells seeded at the same density as previously described and treated with various concentrations of NO-ASA isomers for 48 or 72 h. All three isomers displayed a time- and concentration-dependent reduction in PCNA expression. Although there are individual differences between the three isomers, the general features of their inhibitory effect on PCNA expression are: 1) for each one of them a clear inhibition of PCNA expression becomes evident around their respective IC_{50} concentrations, and 2) at equimolar concentrations, the *ortho*- and *para*-isomers are much more potent than the *meta*-isomer (Fig. 1).

At 48 h compared with control, the *ortho*-isomer started inhibiting PCNA expression at 1 μM, a concentration close to its IC_{50} (15% reduction). This effect was more pronounced at 10 and 100 μM (45 and 57%, respectively). At 72 h, the reduction of PCNA expression by *ortho*-NO-ASA started at even lower concentrations, reaching a plateau at about its IC_{50} (33% reduction compared with control). Similar was the effect of the *para*-isomer, although as can be appreciated

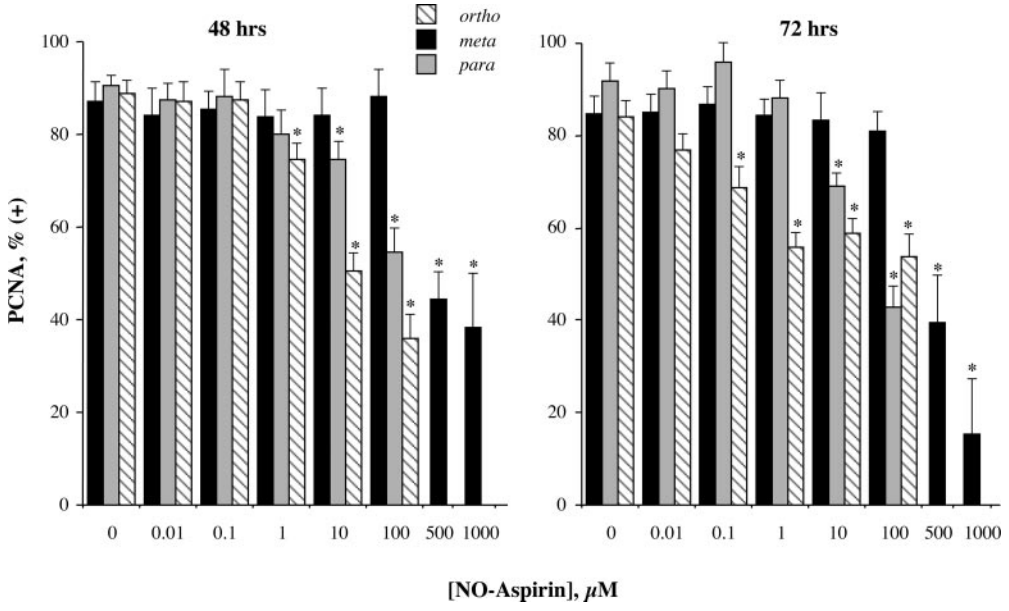


Fig. 1. The effect of the NO-ASA positional isomers on HT-29 colon cancer cell proliferation. Cells were treated with varying concentrations of the *ortho*-, *meta*-, and *para*-isomers of NO-ASA for up to 72 h. PCNA expression was determined by flow cytometry and expressed as percentage positive cells as described under *Materials and Methods*. Results represent mean ± S.E.M. of at least three different experiments with duplicate plates. *, $P < 0.05$ compared with untreated cells.

from the data shown in Fig. 1, there were some differences, especially at 72 h (reduced expression starts at 10 μM). The *meta*-isomer had no effect on PCNA at concentrations up to 100 μM, whereas at 48 h it inhibited it at 500 and 1000 μM (both above its IC_{50}) by 51 and 55%, respectively, whereas at 72 h, it reduced it by 55% at 500 μM and 81% at 1000 μM. Direct comparison of them, e.g., 100 μM at 48 h, reveals that compared with controls, the *meta*-isomer had no effect, *ortho*-NO-ASA reduced PCNA expression by 57% and *para*-NO-ASA by 43%.

The Effect of NO-ASA Positional Isomers on Colon Cancer Cell Death. We observed two types of cell death based on morphological criteria in HT-29 cells treated as above with various concentrations of the three positional isomers (Figs. 2 and 3) One population of cells appeared to have changes consistent with apoptosis: nuclear and cytoplasmic condensation, nuclear fragmentation, and formation of apoptotic bodies. Another population of cells consisted of what we have termed atypical cells (Williams et al., 2001; Kashfi et al., 2002). They maintain their shape but they have either diminished or no nuclear material as detected by DAPI, a dye that stains DNA. These changes have been confirmed by electron microscopy (data not shown, but nearly identical to those already published by us) (Kashfi et al., 2002). The *meta*-isomer induced only apoptotic cells, which never exceeded 50% of the total. The *ortho*- and *para*-isomers induced both forms of cell death. At 24 h, cell death was only apoptotic for all three; at 48 h, apoptotic death predominated (being the only form in *meta*-isomer-treated cells), whereas at 72 h, the atypical cells were the clear majority at concentrations that had a distinct effect on cell growth.

To assess the nature of the cell death induced by NO-ASA, we studied its *meta*- and *para*-isomers in greater detail. We sought to take advantage of the differential staining with annexin and propidium iodide (PI) of early apoptotic versus late apoptotic and necrotic cells. Viable cells are annexin (−) and PI (−). Early apoptotic cells with phosphatidylserine exposed on the outer surface of the plasma membrane but intact cell membranes bind annexin and exclude PI, and necrotic or apoptotic cells in terminal stages are both annexin and PI positive (Nakamura et al., 2002). Purely necrotic cells are annexin (−) and PI (+).

We plated HT-29 cells at a density of 4×10^4 cells/cm^2, treated them with NO-ASA isomers, and harvested them 6,

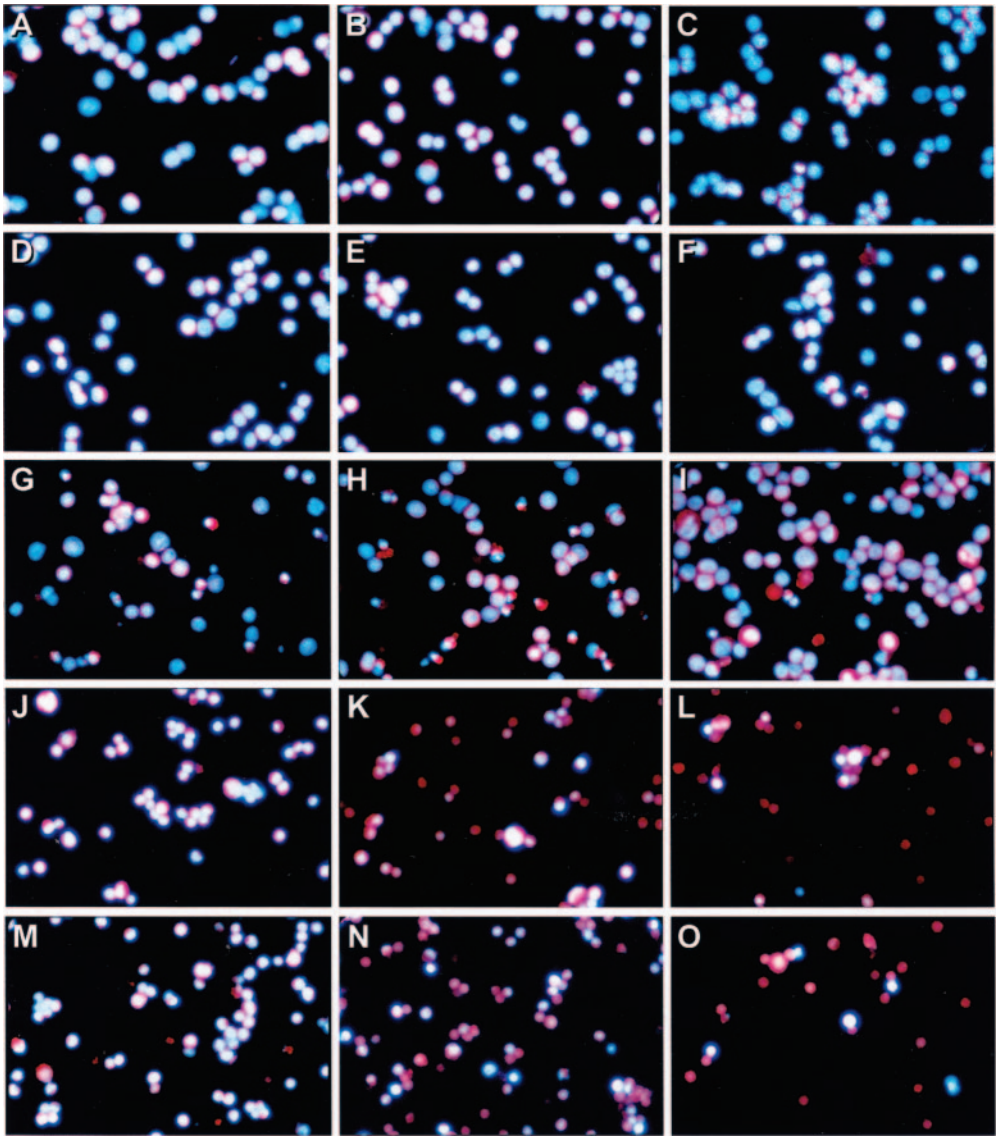


Fig. 2. The effect of the NO-ASA positional isomers on HT-29 colon cancer cell death. Cells treated with 100 μM *ortho*-, *meta*-, and *para*-isomers of NO-ASA, traditional ASA (5 mM), or vehicle for up to 72 h were harvested every 24 h, stained with the DNA stain DAPI, and visualized under fluorescence microscopy. Three populations of cells are evident: unchanged HT-29 cells, apoptotic, and atypical (cells with diminished or nuclear material). A–C, control cells (untreated) at 24, 48, and 72 h. D–F, 5000 μM ASA treated 24, 48, and 72 h. G–I, 100 μM *meta*-NO-ASA treated 24, 48, and 72 h. J–L, 100 μM *para*-NO-ASA treated 24, 48, and 72 h. M–O, 100 μM *ortho*-NO-ASA treated 24, 48, and 72 h.

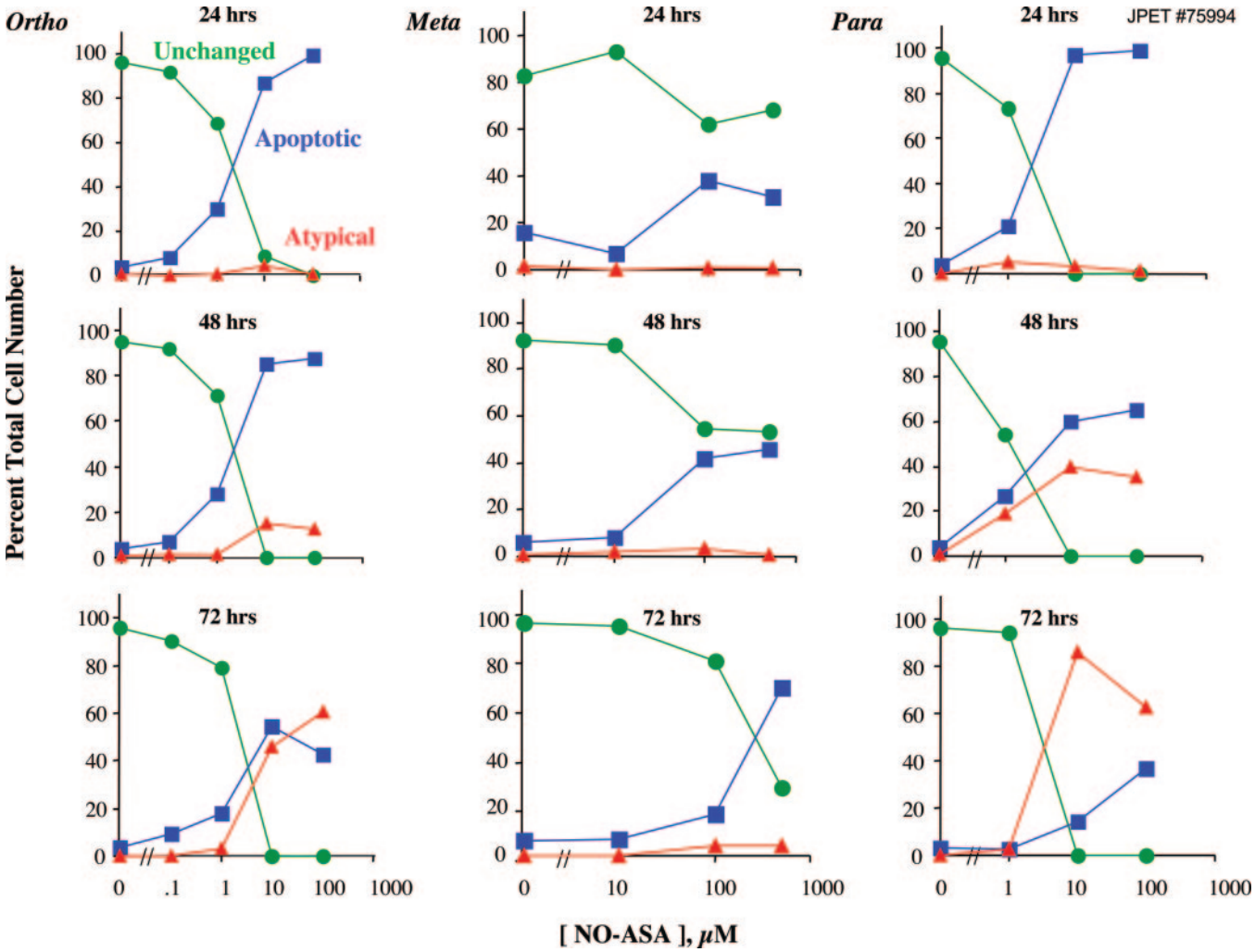


Fig. 3. The kinetic effect of the NO-ASA positional isomers on HT-29 colon cancer cell death. HT-29 cells were treated with various concentrations of the three positional isomers of NO-ASA for up to 72 h and were evaluated morphologically after staining with DAPI as described under *Materials and Methods*. Using photographs similar to those shown in Fig. 2, the cells in each population (unchanged, apoptotic, and atypical) were counted. The results are averages of two different experiments performed in duplicate.

24, or 48 h later. At each time point, these cells were subjected to study by flow cytometry as described above.

As shown in Fig. 4, during the period of observation (6–48 h), 3.4 to 5.3% of untreated (control) cells were apoptotic and 4.6 to 10.9% were late apoptotic/necrotic. Treatment of cells with a low concentration of 10 μM *para*-NO-ASA induced apoptosis, which became apparent at 6 h (double that for control) increasing further at 24 h. The percentage of late apoptotic/necrotic cells was essentially the same as that of the control cells. By 48 h, the percentage of late apoptotic/necrotic cells increased to 23%, whereas apoptotic cells dropped slightly to 21.6%. The highest *para*-NO-ASA concentration used in these studies (100 μM) showed a rapid and quantitatively more dramatic shift of the cell population toward the late apoptotic/necrotic cells. By 6 h, most of the cells (55.8%) had become late apoptotic/necrotic cells. This was further exacerbated at 24 and 48 h, shifting 75.3 and 89% of the cells, respectively, to this category with equal percentages distributed between the other three categories [viable, apoptotic, and purely necrotic, i.e., annexin (−)/PI (+) cells]. Of note, all NO-ASA treatments led to roughly the same percentage of annexin (−)/PI (+) cells, which were 2- to 4-fold greater than baseline and roughly between 2 and 5%.

These data indicate that at low concentrations of *para*-NO-ASA, cells progress through a stage of apoptosis to one of necrosis, characterized by the cell's inability to keep PI out of the cell. In contrast, at high concentrations of *para*-NO-ASA, cells progress rapidly into a stage of advanced necrosis. Within the first 6 h, most cells are unable to exclude PI, and by 24 h, cells that enter this stage of necrosis show no residual membrane viability, as they cannot exclude PI.

The *meta*-isomer of NO-ASA, studied at low (100 μM) and high (1000 μM) concentrations, led to different results. At the lower concentration, this isomer had essentially no effect on the distribution of these cells between the four annexin/PI categories. At the high concentration, the response was reminiscent of that of the high concentration of the *para*-isomer, but with two exceptions: 1) the magnitude of the changes is smaller compared with the *para*-isomer, and 2) by 72 h, there were two prominent cell populations, apoptotic and necrotic, in contrast to the *para*-isomer where essentially all cells were necrotic.

Inhibition of Intestinal Carcinogenesis by the *Meta*- and *Para*-Isomers of NO-ASA. To further assess the biological relevance of our in vitro findings, we determined whether the differential in vitro potency of these compounds

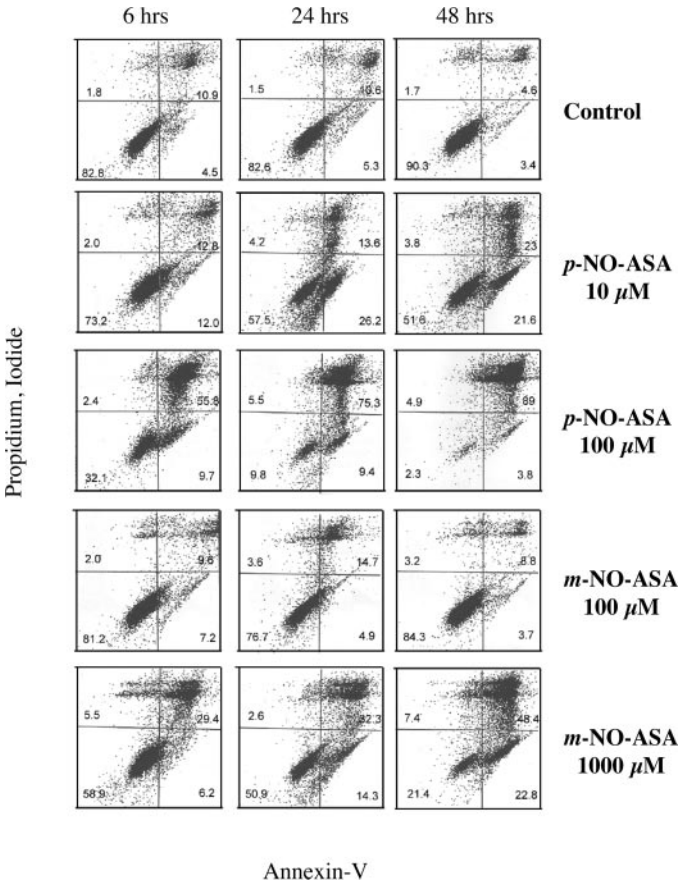


Fig. 4. The effect of *para*- and *meta*-NO-ASA positional isomers on HT-29 colon cancer cell death. Cells were treated with low and high concentrations of the *meta*- and *para*-isomers of NO-ASA for up to 48 h. At each time point, cells were stained with annexin and propidium iodide and subjected to flow cytometric analysis as described under *Materials and Methods*. Numbers represent percentage of cells in each subcategory. All data are from a single experiment. This study has been repeated twice generating results within 10% of those presented here.

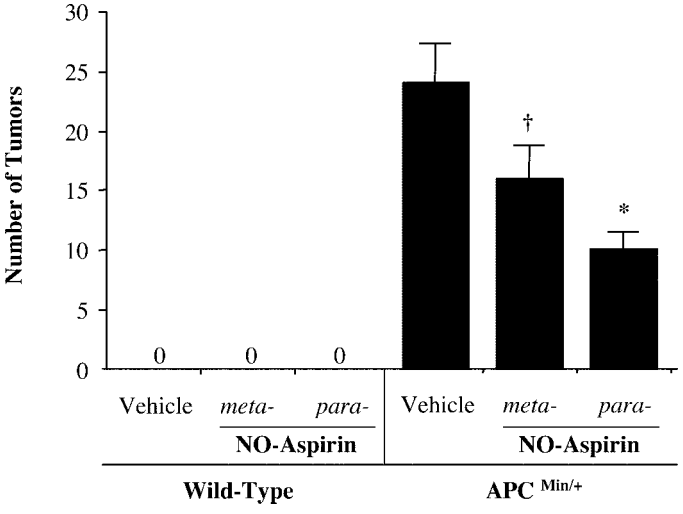


Fig. 5. The effect of *meta*- and *para*-NO-ASA positional isomers on intestinal carcinogenesis in $APC^{Min/+}$ mice. Six-week-old female C57BL/6J $APC^{Min/+}$ mice and the corresponding C57BL/6J$^{+/+}$ wild-type mice were treated with vehicle, *meta*-, or *para*-NO-ASA (each isomer at 100 mg/kg/day) as indicated for 21 days as described under *Materials and Methods*, when the number of small intestinal tumors in each mouse was determined. Values are mean ± S.E.M. (n = 9–10 per group). *, $P < 0.01$; †, $P > 0.05$, statistically not significant.

leads to unequal efficacy on tumor prevention and/or regression in animals. To this end, we determined the effect of equimolar amounts of the *meta*- and *para*-isomers of NO-ASA on 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 (Lipkin et al., 1999). In many important ways, this model system recapitulates the salient steps of colon carcinogenesis and thus represents a useful (and extensively utilized) experimental system.

As shown in Fig. 5, following 3 weeks of treatment with equimolar concentrations of these two positional isomers of NO-ASA, their effect on intestinal tumors in *Min* mice differed. Compared with vehicle-treated controls, the *meta*-isomer decreased the number of tumors in the small intestine by 36% (statistically not significant), whereas the corresponding reduction by the *para*-isomer was 59% ($P < 0.01$). This finding is consistent with the potency ranking of these two isomers based on their effects on cultured cells. Of note, there was no evidence of toxicity in either the *Min* or wild-type mice; the latter, as expected, showed no intestinal tumors.

Structure-Activity Relationships of NO-ASA. We examined the role in the growth inhibitory effect of three critical structural components of the NO-ASA molecule: the $–ONO_2$ group, the spacer, and the acetyl group of the traditional ASA moiety. To this end, we synthesized the structural analogs shown in Table 1.

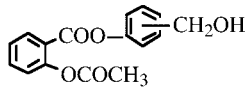
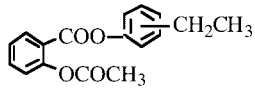
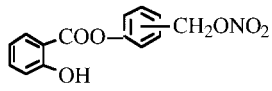
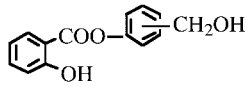
The $–ONO_2$ group is the moiety that is in general considered to be critical for this family of compounds, including NO-ASA. Thus, we synthesized two series of analogs. Those in which the $–ONO_2$ group was replaced by –OH and those in which it was replaced by $–CH_3$. The reason for this was to try to control for the differences in polarity introduced by the new moiety, which could affect access to the cell via its cell membrane. Denitration profoundly diminished the ability of NO-ASAs to inhibit cell growth. However, the analogs bearing the $-CH_3$ group were more potent than those with the -OH group; such a comparison could not be made for the *meta*-isomers as their IC_{50} was indeterminable. Nevertheless, when it could be determined, there was a 3-fold difference in potency between the two. These findings indicate that the $–NO_3$ group (and by extension NO) plays a critical role in the biological effect of NO-ASA.

The deacetylated analogs were used to test the moiety that inactivates COX and accounts for at least some of the biological activity of aspirin that is the structural basis of all three NO-ASA isomers. Deacetylation of all three isomers had no effect on their ability to inhibit cell growth. Their IC_{50} values were virtually identical to those of their parent NO-ASAs. This finding suggests that the effect of NO-ASA on cell growth is not dependent on its ability to acetylate COX.

We then examined whether the simultaneous absence of both the $–ONO_2$ group and the acetyl group had any effect on the ability of these molecules to affect the growth of HT-29 colon cancer cells. We observed that the effect on cell growth was equivalent to that of the molecules that were only denitrated. This indicates, again, that acetylation of COX, or other molecular targets by these molecules, is not required for their effects on colon cancer cell growth.

The spacer is an integral component of the NO-ASA molecule. Its apparent feature is its aromatic nature. Thus, we

TABLE 2
Effect of NO-ASA analogues on the induction of apoptotic and atypical cells

	Ortho			*Meta*			*Para*		
	Unchanged	Apoptotic	Atypical	Unchanged	Apoptotic	Atypical	Unchanged	Apoptotic	Atypical
Denitrated									
0 μM	95.5	3	1.5	92.7	6.3	1	96.1	3.2	0.7
0.01	92.4	7.6	0	-	-	-	-	-	-
0.1	89.1	10	0.9	-	-	-	-	-	-
1	87.7	12	0.3	-	-	-	93.9	5.2	0.9
10	89.3	10	0.7	91.2	8	0.8	93.6	4.9	1.5
100	87.1	12	0.9	83.3	15.2	1.5	94.4	4.5	1.1
1000	-	-	-	15.5	68.6	15.8	-	-	-
Denitrated									
0 μM	97.2	2.1	0.7	97.4	2.6	0	96.1	3.2	0.7
0.01	96.5	1.6	1.9	-	-	-	-	-	-
0.1	81	17.7	1.3	-	-	-	-	-	-
1	39.4	58.8	1.8	-	-	-	93.5	5.7	0.8
10	14.9	79.8	5.3	93.3	6.4	0.3	91.5	6.8	1.7
100	31.3	65.6	3.1	76.1	22.3	1.6	79.4	20.6	0
1000	-	-	-	17	77.7	5.3	-	-	-
Deacetylated									
0 μM	98.5	1.1	0.4	92.7	6.3	1	96	3.3	0.7
0.01	88	11	1	-	-	-	-	-	-
0.1	85	15	0	-	-	-	-	-	-
1	79	20	1	-	-	-	86.8	7.4	5.8
10	0	49	51	94.4	5.6	0	0	73	27
100	0	45	55	65.1	31.6	3.3	0	76	24
1000	-	-	-	16.6	74	9.4	-	-	-
Denitrated / Deacetylated									
0 μM	95.5	3	1.5	92.7	6.3	1	96.1	3.2	0.7
0.01	91.4	7.8	0.8	-	-	-	-	-	-
0.1	91.3	8	0.7	-	-	-	-	-	-
1	97.9	1.8	0.3				94.7	3.8	1.5
10	92.3	7.5	0.2	96.2	3.8	0	94.9	4.9	2
100	92.2	7.8	0	93	6.2	0.8	91.3	7.8	0.9
1000	-	-	-	12.2	79.4	8.4	-	-	-

synthesized an analog in which the aromatic spacer was replaced by an aliphatic one (the nature of the aliphatic spacer led to only one molecule and no isomers). The aliphatic spacer analog displayed greatly reduced ability to inhibit cell growth.

We also examined the effect of these structural analogs of NO-ASA on the induction of apoptosis and the atypical cell, two kinetic parameters with the greatest biological interest (Table 2; data not shown for 24 and 72 h). Compared with the

complete NO-ASA molecules, we obtained the following results.

The *ortho*- and *para*-denitrated analogs bearing the –OH group induced apoptosis only modestly (1.5- to 3-fold higher than control) and failed to induce atypical cells. The *meta*-isomer of this analog had a similar effect at low concentrations and only at 1000 μM did it induce apoptosis and a modest amount of atypical cells.

The *ortho*- and *para*-denitrated analogs bearing the $-CH_3$ group induced significant apoptosis (*ortho* more than *para*) but failed to induce atypical cells. The *meta*-isomer of this analog had roughly a similar effect as the previous *meta*-analog.

The three deacetylated analogs behaved similarly to their parent molecules. The only exception was that the *meta*-isomer induced the atypical cell (nearly 10%) at its highest concentration. It should be noted that even at 1000 μM *meta*-NO-ASA failed to induce atypical cells (data not shown in Fig. 3); at 1000 μM, the solubility of this isomer is limiting. The NO-ASA analog with the aliphatic spacer failed to induce atypical cells (data not shown).

These data suggest that the NO released from these compounds plays an important role in their biological activity. To further explore this possibility, we evaluated the rate of release of NO from each of these positional isomers and from the analog with the aliphatic spacer, the effect of exogenous NO donors on cell growth, and the effect of a NO scavenger on the biological effect of NO-ASA.

We determined the rate of NO release into the culture medium of HT-29 cells by equimolar concentrations (40 μM) of *ortho*-, *meta*-, and *para*-NO-ASA or NO-ASA with an aliphatic spacer; these cells were exposed to these compounds as previously shown. As shown in Fig. 6A, overall the *para*- and *ortho*-isomers released NO at a significantly higher rate than the *meta*-isomer, whereas the NO released from the one with the aliphatic spacer was closer to that of untreated cells. Such NO release depended on NO-ASA concentration (Fig. 6B). To quantify these rates, we calculated the *initial* rate of NO release for each of these compounds based on the amount of NO released during the first 15 min. These rates (nanomoles per minute) were as follows (Fig. 6C, table inset): control = 0.14, *ortho*-NO-ASA = 13.2, *para*-NO-ASA = 8.7, *meta*-NO-ASA = 3.1, and NO-ASA with aliphatic spacer = 1.1. As shown in Fig. 6C, these rates correlated with the 24-h IC_{50} values for cell growth inhibition (2.8, 5, 500, and 900 μM, respectively, confirmed in parallel-run controls); this correlation was statistically significant (one-tailed $P < 0.04$).

The two exogenous NO donors that we studied, SNAP and DETA NONOate, inhibited the growth of HT-29 cells; their respective IC_{50} values were 1000 $\pm$ 57 and 750 $\pm$ 65 μM. When either SNAP or DETA NONOate was combined with traditional ASA, both showed a synergistic effect in terms of cell growth inhibition (Fig. 7). However, the combination of these two molecules failed to reconstitute the potency of NO-ASA. The initial rate of NO release from SNAP was 1.1 μmol/min and for DETA NONOate 7.1 μmol/min, both far higher than that of any NO-ASA. When we combined ASA with 10 nM SNAP, a concentration that mimics closely the rate of NO release by *ortho*-NO-ASA, it enhanced the effect of ASA only marginally.

When hemoglobin, a known NO scavenger, was added to the culture medium prior to adding either NO-ASA or SNAP, their inhibitory effects on cell culture growth were abrogated to a large extent. For example, in the case of *para*-NO-ASA used at its IC_{50} (5 μM), pretreatment with 5 μM hemoglobin increased the number of cells on average by 47% compared with NO-ASA alone. At this concentration, hemoglobin had no effect on cell growth; however, hemoglobin at higher concentrations inhibited cell growth by itself and this precluded experiments attempting complete scavenging of NO. Carboxy-PTIO, another NO scavenger, presented a similar situation (data not shown).

Discussion

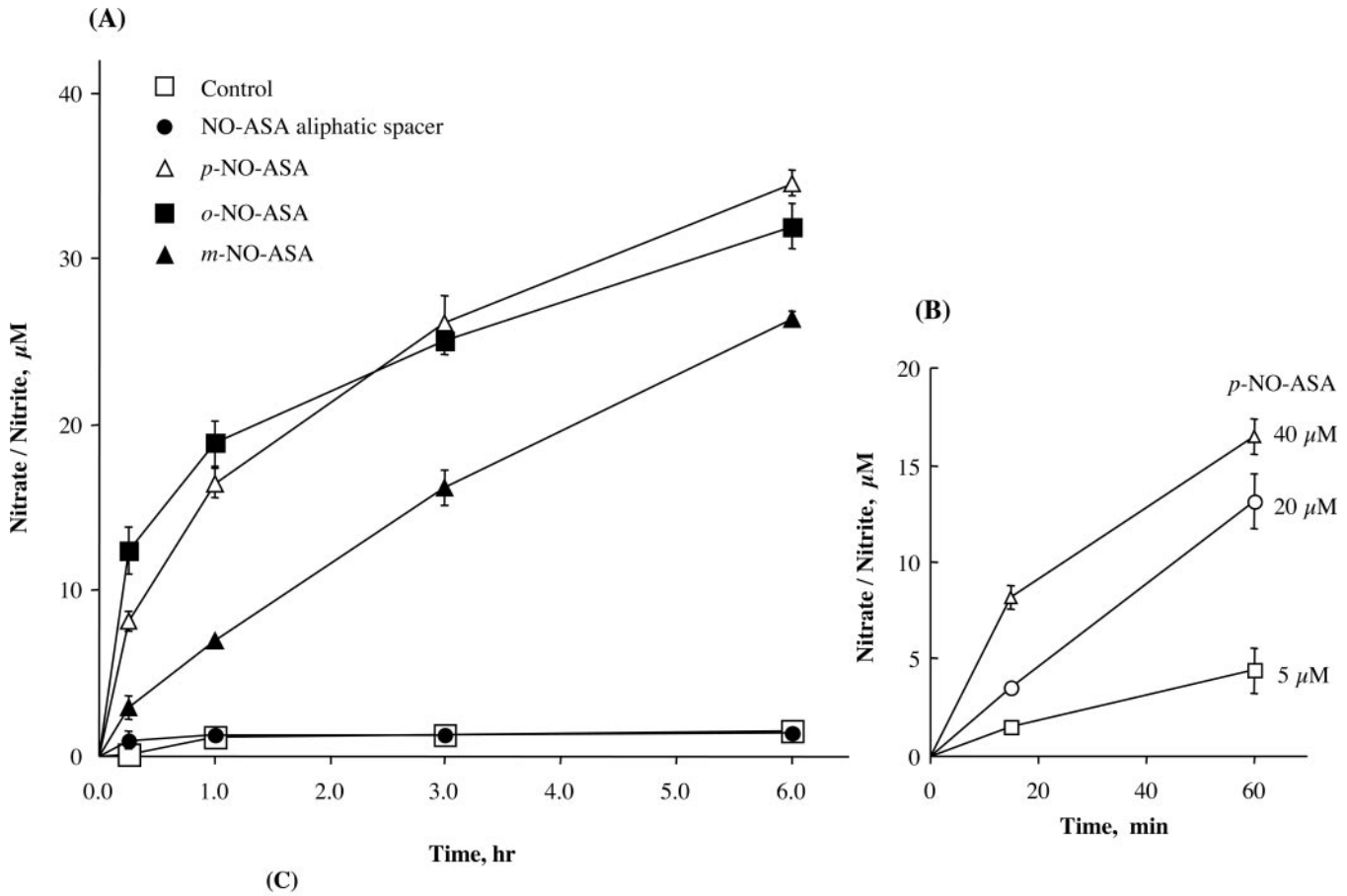
Positional isomerism can at times have a significant effect on the pharmacological properties of a given compound, and here we document such a case for NO-ASA, a promising novel compound. Our findings establish a striking difference in biological activity among the three positional isomers of NO-ASA. This is evident in their ability to inhibit colon cancer cell growth in vitro and the development of intestinal tumors in vivo. Moreover, our data define significant structure activity relationships that bear on the overall effect of NO-ASA on the biology of colon cancer cells.

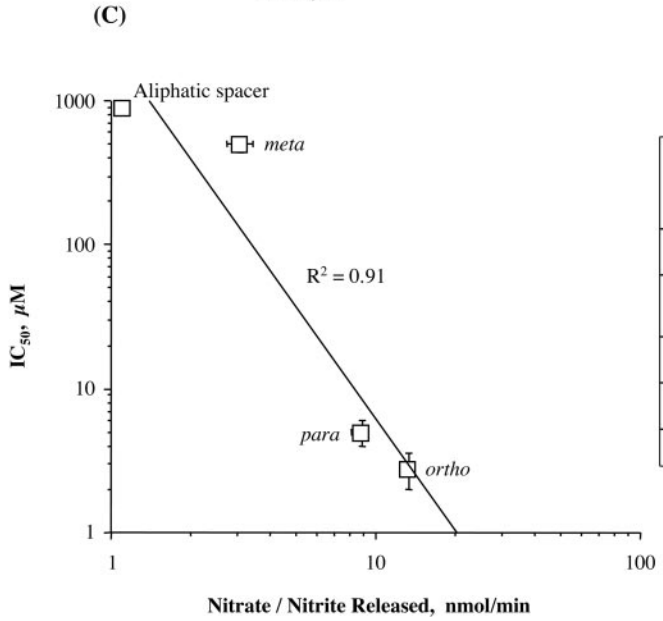
There is a clear separation between the three isomers in terms of their ability to inhibit colon cancer cell growth; two isomers, the *ortho* and *para*, behave similarly but both act quite differently from the *meta*-isomer. Their concentration-response curves and the IC_{50} values derived from them illustrate this point clearly. The IC_{50} values for the *ortho*- and *para*-isomers over 72 h of observation ranged between 1 and 4.8 μM and between 2 and 5 μM, respectively. In contrast, the corresponding IC_{50} values for the *meta*-isomer ranged between 200 and 500 μM. Overall, the *ortho*- and *para*-isomers were roughly 100 to 264 times more potent than the *meta*-isomer in inhibiting the growth of HT-29 colon cancer cells.

The cell kinetic effect of these three compounds, the next level of complexity, maintained this separation between the *ortho*- and *para*-isomers on one hand and the *meta*-isomer on the other. All three isomers inhibited cell proliferation, and for each one of them, a clear inhibition of PCNA expression became evident around their respective IC_{50} concentrations. This finding underscores the important contribution of the antiproliferative effect of these compounds to cell growth inhibition. What distinguishes them, however, is that the *ortho*- and *para*-isomers, similar to each other, are much more potent than the *meta*-isomer when compared at equimolar concentrations.

The effect of these compounds on cell death also reflects this clear separation between these isomers. The *ortho*- and *para*-isomers are roughly equipotent in inducing cell death and much more potent than the *meta*-isomer. A very interesting finding has been the ability of both *ortho*- and *para*-NO-ASA to induce an accelerated progression of the cell to a form of cell death that we have provisionally termed "atypical"; the *meta*-isomer failed to induce this form of cell death, even at the highest concentrations that we could use (1 mM, a concentration that is at the limit of its solubility in culture media).

Morphological and biological data (membrane changes including changes in its permeability to a reporter molecule) combined with our previous observations (Kashfi et al., 2002)

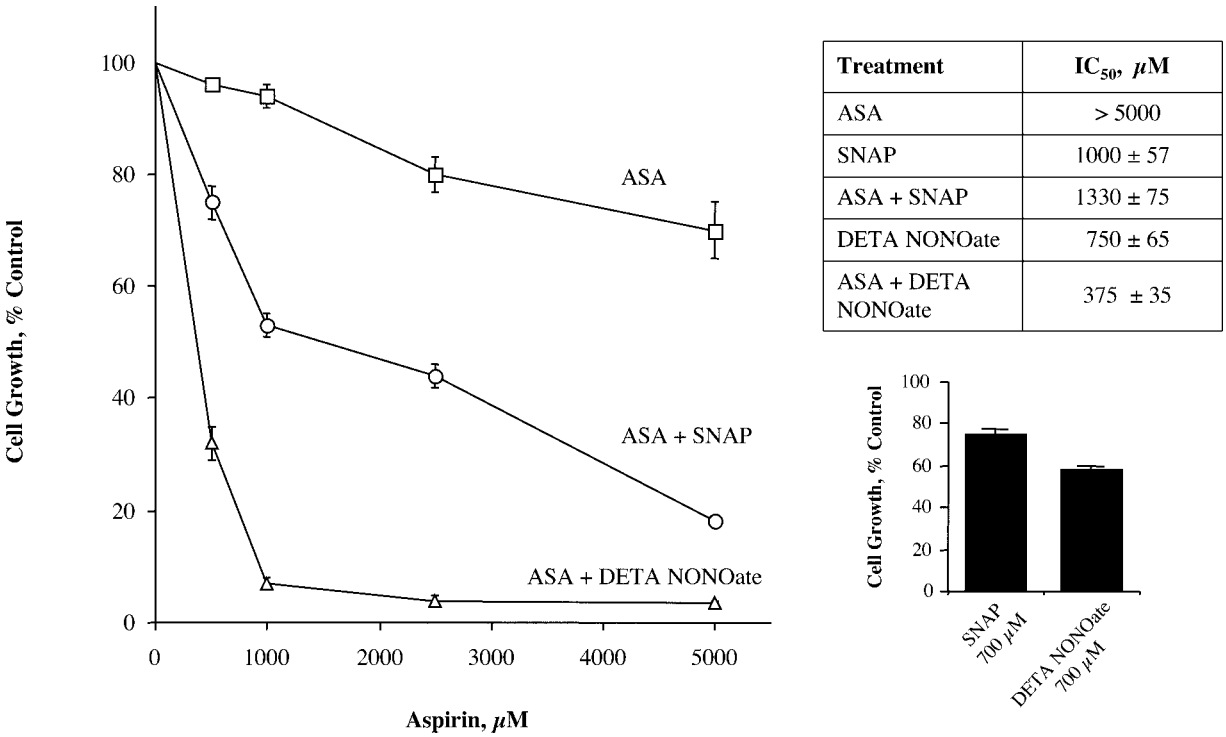




Treatment	Nitrate/nitrite rate of release *nmol/min*
None (control)	0.14 ± 0.03
NO-ASA with aliphatic spacer	1.1 ± 0.1
m-NO-ASA	3.1 ± 0.2
p-NO-ASA	8.7 ± 0.5
o-NO-ASA	13.2 ± 0.7

Fig. 6. Nitric oxide released from NO-ASA. A, HT-29 cells were treated with each one of the three positional isomers of NO-ASA or the one having an aliphatic spacer, and the levels of NO in the culture medium were determined as described under *Materials and Methods*. B, NO release depended on NO-ASA concentration (data shown are for *para*-NO-ASA). C, the initial rate of NO release by the various NO-ASAs correlates with their cell growth inhibitory effect (IC_{50} at 24 h).

indicate that the atypical cell is a necrotic cell. Analysis of the temporal sequence of the appearance of this cell indicates that the *para*-isomer (the *ortho* was not studied in detail) induces a range of changes that include classical apoptosis (minor effect) and various degrees of necrosis (dominant effect). This effect is concentration-dependent and appears to be rapid, especially at high concentrations. The *meta*-isomer behaves similarly but with three key differences. The magnitude of the effect is smaller but slower; the induction of apoptosis is more pronounced with the extent of necrosis limited proportionally; and its necrotic cells do not have the morphological features of the so-called atypical cells. In fact,



Treatment	IC_{50}, μM
ASA	> 5000
SNAP	1000 ± 57
ASA + SNAP	1330 ± 75
DETA NONOate	750 ± 65
ASA + DETA NONOate	375 ± 35

Fig. 7. Cell growth inhibition by ASA and NO donors. HT-29 cells were treated for 24 h with various concentrations of ASA alone, NO donors (700 μM SNAP or 700 μM DETA NONOate) alone, or ASA combined with either NO donor. The combined application of ASA with either NO donor led to a synergistic effect on cell growth inhibition.

in the case of the *meta*-isomer, we identified these cells as necrotic based on the flow cytometric study that revealed their inability to exclude membrane permeability reporter molecules. It is conceivable that the *ortho*- and *para*-isomers induce changes in the cell that lead rapidly to its necrotic death, whereas changes induced by the *meta*-isomer lead at a relatively slower pace to apoptosis, which culminates into cell necrosis (secondary necrosis). We are currently studying these effects in detail.

These differences in potency, obtained in a cell culture system, cannot be extrapolated a priori to animal systems. This is the reason why we assessed their validity in an animal model system of colon cancer. *Min* mice offered such a model (Lipkin et al., 1999). The *para*-isomer was twice as potent as the *meta*-isomer in reducing the number of intestinal tumors in these mice, even after a relatively brief treatment period. This confirms our basic observation that positional isomerism is indeed an important determinant of NO-ASA's pharmacological activity.

The structure-activity study underscores the complexity of the effect of NO-ASA on cancer cell growth. There are at least four important findings from this study. First, the $-ONO_2$ group, which defines this new class of agents as NO-donating compounds, is critical for its biological activity in these colon cancer cells, but its precise contribution to the overall effect remains unclear and deserves further detailed study. Nevertheless, our data provide a first approximation to assessing this issue.

The three isomers and the analog with the aliphatic spacer release NO at different rates. Remarkably, there is an excellent correlation between the initial rate of NO release by the four NO-ASA molecules (*ortho*-, *meta*-, *para*-, and the one with the aliphatic spacer) and their cell inhibitory potencies, as measured by their IC_{50} values. This finding underscores the contribution of NO to the biological effect of NO-ASA. That NO is important in this respect is further supported by two additional findings: 1) exogenous NO donors inhibited cell growth, although with higher IC_{50} values than NO-ASA, and 2) NO scavenging partly reversed the cell growth inhibitory effect of NO-ASA. (Complete scavenging was not feasible, as the various NO scavengers that we tried had their own intrinsic cell inhibitory properties.)

The underlying mechanism, however, appears more complex, since our experiment attempting to "reconstitute" two principal components of this molecule, ASA and NO, using exogenous NO donors did not reproduce the potency of NO-ASA, even though in both cases there was a synergistic effect between them. This finding should be interpreted with caution, however, as what we measured were NO levels in the media and not intracellular levels. Thus, it is conceivable that the entire NO-ASA molecule delivers NO more efficiently at sites where it is biologically effective, and the NO levels in the media do not necessarily reflect NO concentrations at specific cellular sites. An example of such a situation is that of p53 mutations by NO in colon cancer. The inducible nitric-oxide synthase (NOS2), overexpressed in colon cancer, locally generates high NO concentrations which damage the nearby DNA (e.g., *p53* gene), but dissipate as the molecule diffuses away from its site of production (Ambs et al., 1999).

Regardless of these findings, the $-ONO_2$ group is likely not the sole determinant of the biological activity of NO-ASA. This notion is supported by the not insignificant growth inhibitory effect of the analogs bearing the $-CH_3$ group. It is tempting to speculate that the lipophilicity of these analogs facilitates their access to the cell through the cell membrane,

thus revealing the residual biological activity of NO-ASA after it was deprived of its "NO-donating" moiety.

Second, the spacer is also important for NO-ASA's effect. Substituting the aliphatic spacer for the aromatic one brought the potency in growth inhibition of NO-ASA to about half that of the *meta*-isomer.

Third, the acetyl group of NO-ASA had practically no bearing on the biological activity of NO-ASA. This observation agrees with a series of reports that cast doubt as to whether COX is the sole molecular target of NSAIDs or compounds that share NSAID features. The notion of COX-independent effects in cancer prevention is now fairly well accepted, and our current data are in accord with it (Rigas and Shiff, 2000).

What remain less clear are the reasons inherent in these positional isomers for such a differential effect and the cell pathways by which this unusual differentiation is brought about. As explained in the following article, the three isomers are metabolized differently in terms of both rates of biotransformation and end products (Fig. 10 of that article). Interestingly, the *ortho*- and *para*-isomers behave similarly and in a way that is distinctly different from that of the *meta*-isomer. This difference may account, at least in part, for their differences in biological effect. In terms of cellular effects, the differences in cell death induction between the *ortho*- and *para*-isomers on the one hand and the *meta*-isomer on the other were clear, especially regarding the atypical cells (the first two generate them, the latter does not). However, the fact that all *meta*-analogs induced atypical cells, albeit to a limited degree, brings into question whether this effect could reveal a defining mechanistic difference between these two groups of positional isomers.

In conclusion, our work provides clear evidence that positional isomerism influences the NO-ASA molecule in a biologically important way with regard to the inhibition of colon cancer cell growth in vitro and intestinal carcinogenesis in vivo. We have identified mechanistically critical components of the NO-ASA molecule with the $-ONO_2$ group appearing most important, whereas the differential induction of the atypical cell may be part of "downstream" differences between the three positional isomers. This work suggests the need to further study this phenomenon, which may have important implications not only for cancer prevention and treatment using this class of drugs, but also for the rational design of drugs in general.

Acknowledgments

We thank Dr. Xiaopeng Liu for technical assistance and useful discussions.

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