

The differential cell signaling effects of two positional isomers of the anticancer NO-donating aspirin

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Abstract. We studied the mechanism by which the *para* and *meta* positional isomers of nitric oxide-donating aspirin (NO-ASA) inhibit human colon cancer cell growth. These compounds are promising chemopreventive agents and represent a broader class of novel drugs. The two isomers differ drastically in their 24-h IC₅₀s for cell growth, which are 12 μ M for *p*-NO-ASA and 230 μ M for *m*-NO-ASA. We examined their effects on cell signaling cascades, including predominantly the mitogen activated protein kinases (MAPKs). The principal differences between the two isomers were: a) *p*-NO-ASA exerts its effect earlier than *m*-NO-ASA; b) the predominant effect of *m*-NO-ASA is on ERK1/2 and Akt; whereas that of *p*-NO-ASA is on JNK1/2, while both activate p38, with *p*-NO-ASA showing a stronger and earlier effect; c) ATF-2 is more responsive to *m*-NO-ASA and c-Jun to *p*-NO-ASA; d) both isomers seem to have similar effects on AP-1 binding, the main difference between them being the timing of the effect; *p*-NO-ASA's effect is early and *m*-NO-ASA's is late; e) *p*-NO-ASA has an earlier and stronger effect on p21, while *m*-NO-ASA's effect occurs later and is weaker; and f) cell cycle changes follow the effect on p21 expression. Our findings underscore the role of positional isomerism in modulating the pharmacological effects of drugs and have potentially important implications for the further development of these chemoprevention agents.

Introduction

Colon cancer has the third highest cancer incidence in the United States, with over 50,000 individuals dying each year of causes related to colorectal cancer (1). These statistics reflect the limitations of currently available treatments. The development of new preventive protocols is essential to reduce the incidence of colon cancer. In the search of such approaches, aspirin and other non-steroidal antiinflammatory agents have shown promising results (2-4). The prolonged use of aspirin for cancer prevention in doses other than those used for cardiac prophylaxis, however, is not practical because of its toxicity (5,6). Thus, new anti-inflammatory derivatives of aspirin, such as nitric oxide-donating aspirin (NO-ASA), are being explored. NO-ASA shows much promise because of its significantly lower gastrointestinal toxicity compared to aspirin (7) and its antineoplastic effects in preclinical models of colon cancer (8-10); for example, NO-ASA inhibits the growth of cultured pancreatic and colon cancer cells (8,11), as well as intestinal carcinogenesis in *Min* mice (9) and pancreatic cancer in hamsters (12).

Isomerism is of particular interest to pharmacology as it can influence drug activity, at times profoundly (13-15). There are four main types of drug isomers, positional isomers, geometric isomers, enantiomers and diastereoisomers. Positional isomers are compounds of the same molecular formula but differ in the position of a functional group in the drug molecule. The ability of positional isomerism to modulate drug effect has been classically exemplified by the isomeric amobarbital and pentobarbital, in which the position of a methyl group markedly affects their pharmacological activity. NO-ASA has three positional isomers *para*, *meta* and *ortho*, based on the position of the -ONO₂ group with respect to its ester bond (Fig. 1). We have reported that these positional isomers vary in their potency to inhibit the growth of colon cancer cells; *ortho* and *para* are nearly equipotent, whereas the *meta* is far less potent than the other two (16). We have explored several intracellular signaling pathways to determine how *p*-NO-ASA mediates its unique growth inhibitory effects (10,17-21). Previously, we identified mitogen activated protein kinase (MAPK) pathways as mediators of *p*-NO-ASA's growth inhibitory effect (19). Employing two colon cancer and one pancreatic cell line we demonstrated that *p*-NO-ASA activated several MAPKs, and that JNK and p38 were required for its growth inhibitory effect (19).

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Abbreviations: EMSA, electrophoretic mobility shift assay; MAPKs, mitogen activated protein kinases; NO-ASA, nitric oxide-donating aspirin

Key words: nitric oxide-donating aspirin, positional isomerism, p21, mitogen activated protein kinases, cancer prevention

The present study examined whether the significant difference in the growth inhibitory potency of *m*- and *p*-NO-ASA could be accounted for by differences in their intracellular signaling. Thus, we compared the signaling cascades activated by the isomers of NO-ASA, focusing on the MAPK signaling cascade and its dependent pathway that leads via p21 to cyclin D1 expression and cell cycle regulation. We demonstrated significant differences between the two isomers of NO-ASA in MAPK signaling, which led to differential induction of p21, which in turn affected cyclin D1 expression and cell cycle phase transitions.

Materials and methods

Materials. NO-ASA, both *para* [2-(acetyloxy) benzoic acid 4-(nitrooxy methyl) phenyl ester] and *meta* [2-(acetyloxy) benzoic acid 3-(nitrooxy methyl) phenyl ester], was synthesized as previously described (22). Antibodies against the phosphorylated forms of cJun, ATF-2, JNK, p38, ERK1/2, Akt and total p21 were purchased from Cell Signaling Technology, Beverly, MA. Horseradish peroxidase conjugated antibodies against α -tubulin, mouse IgG, or rabbit IgG were purchased from Oncogene Research Products, San Diego, CA. All siRNAs were obtained from Upstate Cell Signaling Solutions, Lake Placid, NY. Cell lines were obtained from American Type Culture Collection (ATCC), Manassas, VA and cell culture reagents from Mediatech, Herndon, VA.

Cell culture and treatments. Cells were grown to 80% confluence in the media recommended by ATCC supplemented with 10% fetal calf serum, 1,000 U/ml penicillin and 1,000 μ g/ml streptomycin. For treatment with various reagents, 1×10^6 cells/plate were plated in 6-well plates (Corning, Corning, NY). NO-ASA and other reagents were incubated with cell monolayers in the presence of 1% DMSO in culture medium. After incubation, cultures were placed on ice, the medium was collected and cells were washed twice with phosphate-buffered saline (PBS). Lysis buffer [20 mM HEPES, 50 mM NaF, 1 mM Na_3VO_4 , pH 7.3, with 10% (v/v) glycerol, 1% (v/v) Triton X-100, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 1 mM PMSF, and 2.5 mM 4-nitrophenylphosphate] was added to cultures in wells; floating cells from the cultures were pooled with the adherent cells. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL).

Cell proliferation assay. Cell proliferation was assayed using the MTT colorimetric assay kit (Roche, Germany), following the manufacturer's instructions.

Western blots. Electrophoresis of cell lysates were performed on 10% SDS-PAGE gels as described (23); protein were electrophoretically transferred onto nitrocellulose membranes, which were reprobed as needed.

Isolation of nuclear extracts and electrophoretic mobility shift assays (EMSA). Nuclear extracts from cells cultured as above, were obtained as described (24), with the modifications listed below. Cells (approximately 1×10^7 /100 mm dish) were incubated on ice with whole cell fractionation buffer [10 mM HEPES, pH 7.9, 50 mM NaF, 1 mM Na_3VO_4 , 0.1 mM EDTA,

0.5 mM DTT, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 250 mM 4-nitrophenylphosphate and 1 mM PMSF] for 15 min, then scraped and placed in chilled 1.5 ml microfuge tubes on ice. To each 1.5 ml suspension 0.15 ml of 10% (v/v) NP-40 was added and the suspension was gently mixed. Tubes were centrifuged for 1 min at 4°C, the supernatant was aspirated, 0.05 ml of nuclear extraction buffer [20.0 mM HEPES, pH 7.9, 25 mM NaF, 10 mM Na_3VO_4 , 0.1 mM EDTA, 1.5 mM MgCl_2 , 420 mM NaCl, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, 250 mM 4-nitrophenylphosphate, 1 mM PMSF and 25% (v/v) glycerol] was added, incubated for 30 min on a rocker with sporadic vortexing and centrifuged at 16,168 \times g for 10 min at 4°C. The supernatants were collected as nuclear extracts, aliquoted and stored at -80°C until analyzed.

EMSA. EMSA for transcription factor binding to the AP-1 site was performed using a non-radioactive EMSA kit from Panomics, Redwood, CA. Nuclear extracts (10 μ g) were incubated with an AP-1 botinylated probe for 30 min at room temperature. Excess cold AP-1 probe was added as a control. Separation of bound from unbound oligonucleotide probe was performed by electrophoresis in non-denaturing polyacrylamide gels and complexes were transferred to a nylon membrane. Biotin-labeled probe was visualized by enhanced chemiluminescence (ECL). The identification of transcription factors bound to the AP-1 probe was achieved by reprobing the nylon membranes with antibodies directed against cJun, ATF-2 and JunB to determine which transcription factors were bound to the AP-1 probe. This method was verified by incubating the nuclear extracts with a nonbiotinylated AP-1 probe and electrophoretic separation of complexes. The separated complexes were transferred to nylon membranes and the membranes were probed with antibodies to various transcription factors.

Cell transfections with siRNA. BxPC-3 cells in 6-well plates at 80% confluence were transfected with 200 ng of a given siRNA suspended in 100 μ l Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 48 h post transfection, transfected cells were replated and treated with NO-ASA.

Evaluation of cell cycle progression. Cells (2.0×10^6 cells) were incubated in the presence of *m*- or *p*-NO-ASA or equivalent volumes of DMSO for 4 and/or 24 h. After each treatment, cells were trypsinized and fixed in 70% (v/v) ethanol for 1 h on ice, stained with propidium iodide (PI) (50 μ g/ml) and RNase A (4 U/ml) for 30 min and subjected to flow cytometry analysis.

Statistical analysis. Statistical evaluation of the data was performed by one-factor analysis of variance followed by Tukey test for multiple comparisons. $P < 0.05$ were regarded as statistically significant. Data presented were obtained from at least 3 independent experiments ($n=3$) and are expressed as the mean \pm SEM.

Results

Both isomers of NO-ASA inhibit the growth of colon cancer cells. HT-29 cells were incubated with *para* or *meta* NO-ASA

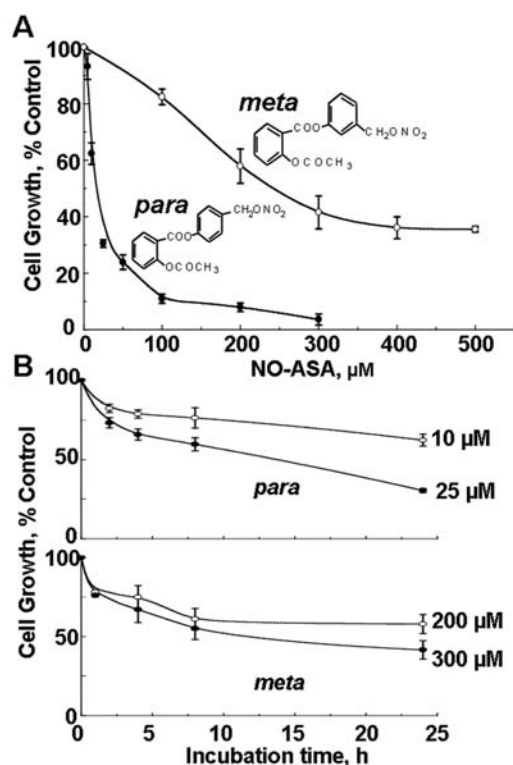


Figure 1. Effects of *para* and *meta* isomers of NO-ASA on cell growth. (A) HT-29 colon cancer cells were incubated for 24 h with various concentrations of either the *para* (black dots) or the *meta* (white dots) isomer of NO-ASA. Cell growth was determined by the MTT cell proliferation assay following the manufacturer's protocol. The structures of the positional isomers are shown next to the isomer's name. (B) Cells incubated for 1, 4, 8 and 24 h with concentrations below and above the IC_{50} for each isomer of NO-ASA. Data are expressed as the percent of untreated control cultures and standard error bar represent the standard error of the mean (SEM) for at least three separate experiments.

for up to 24 h. Shown in Fig. 1A, are the dose response curves for growth inhibition by these isomers of NO-ASA. The IC_{50} s for the *para* and *meta* isomers differ by about 20-fold, being 12 ± 5 and 230 ± 15 μ M, respectively. As shown in Fig. 1B, the time-response curves of both isomers follow a similar course: after an initial strong decline during the first 4 h, there is a shallow decrease in the number of viable cells. Although the reason for this biphasic response is unclear, it is conceivable that it reflects the rate of metabolic transformation of NO-ASA (22).

The effect of *m*- and *p*-NO-ASA isomers on MAPK activation. Treatment of colon cancer cells with *m*- or *p*-NO-ASA stimulates MAPK phosphorylation over the 24 h incubation period. The effects we obtained, depicted in Fig. 2, can be summarized as follows.

At 1 h, *p*-NO-ASA increased the phosphorylation of ERK1/2 MAPK significantly only at concentrations above its IC_{50} for cell growth, while at the concentrations used the *meta* isomer only demonstrated increased phosphorylation of ERK1/2 after 24 h treatment. Akt was marginally, if at all, affected by *p*-NO-ASA. At IC_{50} concentrations only the *meta* isomer significantly affected the phosphorylation of Akt, increasing it at 4 and 8 h. The level of phosphorylated Akt returned to basal levels by the end of the 24 h incubation.

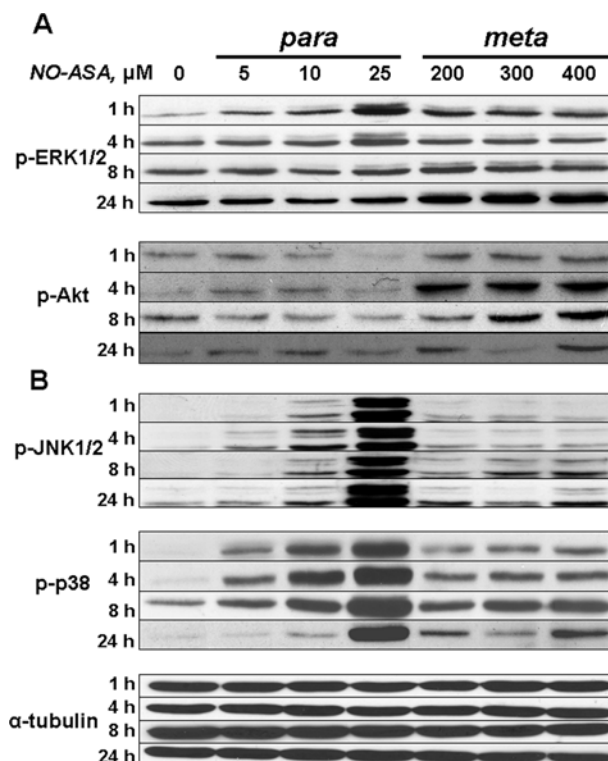


Figure 2. Effects of *para* and *meta* isomers of NO-ASA on MAPKs phosphorylation. HT-29 cells were treated with the indicated concentrations of each NO-ASA isomer for 1-24 h. Western blots of the phosphorylated forms of (A) ERK1/2 and Akt and (B) JNK and p38. α -tubulin is shown as a loading control. For each time point comparisons should be made against the control (0) which was electrophoretically separated on the same polyacrylamide gel and transferred to the same membrane as the treated lysates for the same incubation period. Representative Western blots of more than five experiments are shown.

Both isomers of NO-ASA significantly increased the phosphorylation of the two stress-MAPKs, JNK and p38. Increased phosphorylation of JNK1/2 was observed within 1 h after treatment with *p*-NO-ASA and peaked at 4 h, being approximately 10-fold higher than the basal level. Between 8 and 24 h, the *para* isomer sustained an increased level of phosphorylated JNK of 2-3-fold over the basal level. At a concentration of 25 μ M the *para* isomer stimulated JNK phosphorylation to levels that were more than 20-fold over basal phosphorylation. *m*-NO-ASA increased JNK phosphorylation, which peaked 8 h after treatment was initiated and was 3-fold higher than the basal level. This phosphorylation was sustained for the entire period of observation (24 h).

Both isomers of NO-ASA stimulated large increases in the level of phosphorylated p38, starting as early as within 1 h of treatment. Similar to the stimulated phosphorylation of JNK, the *para* isomer at 25 μ M enhanced p38 phosphorylation by more than 20-fold, maintaining that high level from 1-24 h. Compared to control baseline, *p*-NO-ASA 10 μ M (approximately at its IC_{50}) stimulated p38 phosphorylation 8-fold, an effect that peaked at 4 h, slowed to 3-fold over basal at 8 and at 24 h the level of phosphorylated p38 returned to near basal. Compared to control, the *meta* isomer at its IC_{50} increased p38 phosphorylation about 3-fold, peaking at 8 h. As was observed with the *para* isomer, *m*-NO-ASA at

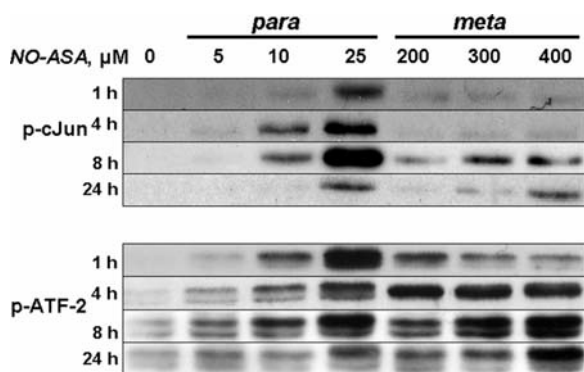


Figure 3. Effects of *para* and *meta* isomers of NO-ASA on the phosphorylation of MAPK transcription factors. HT-29 cells were treated with the indicated concentrations of each NO-ASA isomer for 1–24 h. Western blots of the phosphorylated forms of cJun and ATF-2. For each time point, comparisons should be made against the control (0) which was electrophoretically separated on the same polyacrylamide gel and transferred to the same membrane as the treated lysates for the same incubation period.

approximately twice its IC_{50} stimulated the prolonged phosphorylation of p38 that remained 5 to 8-fold higher than its basal levels.

Given that the activation of JNK and p38 MAPKs leads to the activation of the downstream transcription factors cJun and ATF-2, we next evaluated if the isomers of NO-ASA activated these transcription factors. At 1 h, *p*-NO-ASA 25 μ M activated cJun. This increased at later time points, becoming evident even at lower concentrations and peaking at 8 h. At its highest (8 h, 25 μ M *p*-NO-ASA), phosphorylated cJun was 10–12-fold higher than its basal level (Fig. 3). In contrast, the *meta* isomer at about its IC_{50} increased the phosphorylation of cJun much less and later than the *para* isomer (3-fold increase over baseline at its peak, which occurred at 8 h). Both the *para* and *meta* isomers of NO-ASA at concentrations above their respective IC_{50} s maintained some residual cJun phosphorylation at 24 h.

Both NO-ASA isomers phosphorylated ATF-2 early (within 1 h) and fairly strong. As shown in Fig. 3 and 9, at concentrations approximately their IC_{50} values, the activation of ATF-2 in HT-29 cells was similar for both isomers, whereas at much higher concentrations, the *para* isomer (25 μ M) caused a strong and early activation of ATF-2 with *meta* (400 μ M) showing a later response, which appeared quantitatively similar to that of *para*.

In BxPC3 cells (which, in contrast to HT-29, could be transfected with siRNA), the strong activation of ATF-2 in response to *m*-NO-ASA appears to be partially regulated by p38 and JNK; silencing p38 or JNK or both reduces by about half the induction of ATF-2 by *m*-NO-ASA (Fig. 4A). Interestingly, *p*-NO-ASA 10 μ M does not induce ATF-2 in BxPC3 cells. Both *m*- and *p*-NO-ASA induced the expression of c-Jun. Silencing JNK or JNK in combination with p38 virtually eliminated the induction of c-Jun by both NO-ASA isomers (Fig. 4B).

The effect of NO-ASA positional isomers on AP-1-DNA binding. We investigated if the differences in cJun and ATF-2 activation by the isomers of NO-ASA result in differences in the binding of these transcription factors to the AP-1 consensus

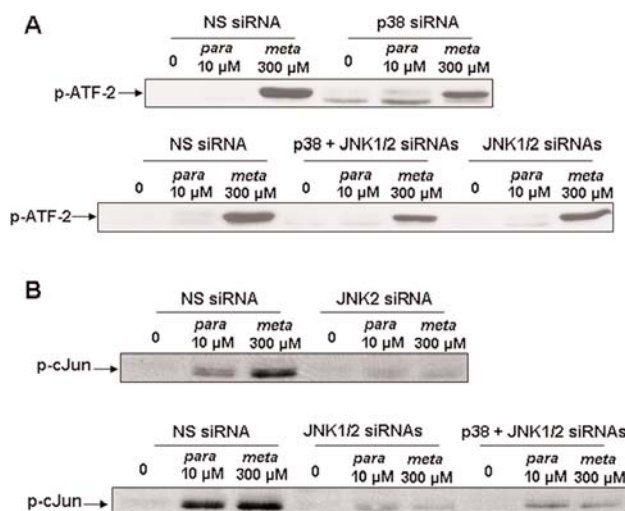


Figure 4. siRNA directed against JNK and p38 affects the activation of ATF and cJun by either isomer of NO-ASA. BxPC-3 cells were transfected with siRNA directed against p38 and/or JNK1/2 mRNA as described under Materials and methods before Western blotting. siRNA-treated and control cells were treated with *para* NO-ASA or *meta* NO-ASA (10 or 300 μ M, respectively). Western blots for p-ATF-2 (A) and p-cJun (B) are shown. Representative Western blots of at least three independent experiments are shown.

sequence using EMSA. Supershift assays demonstrated that in these cells the AP-1 complex consisted of cJun and ATF-2 (data not shown). Both NO-ASA isomers increased the AP-1-DNA binding. In keeping with the trend we have observed, the maximum AP-1-DNA binding effect for *p*-NO-ASA and *m*-NO-ASA was observed at 4 and 8 h, respectively (Fig. 5B). The EMSA blots were compared to Western blots of whole cell lysates from parallel cultures probed for the transcription factors. Interestingly, the binding of transcription factors in nuclear extracts to the AP-1 probe correlated with the phosphorylation level of cJun and ATF-2 (Fig. 5B).

The effect of NO-ASA positional isomers on p21 expression and cell cycle progression. A significant downstream effector of MAPKs is p21, a CDK inhibitor that limits proliferation by inhibiting cyclin-CDK complexes (25). We examined the effect of both isomers of NO-ASA on p21 levels in HT-29 cells. Both compounds induced the expression of p21 in a time- and concentration-dependent fashion (Fig. 6). In the case of *p*-NO-ASA, a strong and significant induction of p21 was noted at 4 h, which dissipated by the end of the 24 h incubation. On the other hand, the *meta* isomer stimulated a much weaker increase in the levels of p21 that became apparent at 8 h and persisted at similar levels up to 24 h (Fig. 6).

We next analyzed the effect of each NO-ASA isomer on the cell cycle distribution of HT-29 cells. At an early time point (4 h), and consistent with the observed increase in p21 levels, only *p*-NO-ASA affected the cell cycle, blocking the S-to-G₂/M transition (Table I). At 24 h, when the effect of *p*-NO-ASA on p21 had essentially disappeared only a slight perturbation in the cell cycle was detected. The *meta* isomer, which showed no effect on p21 or the cell cycle during the first 4 h, blocked the G₂/M-to-G₁ transition at 24 h (Table I), following the induction of p21 that started at 8 h (Fig. 6).

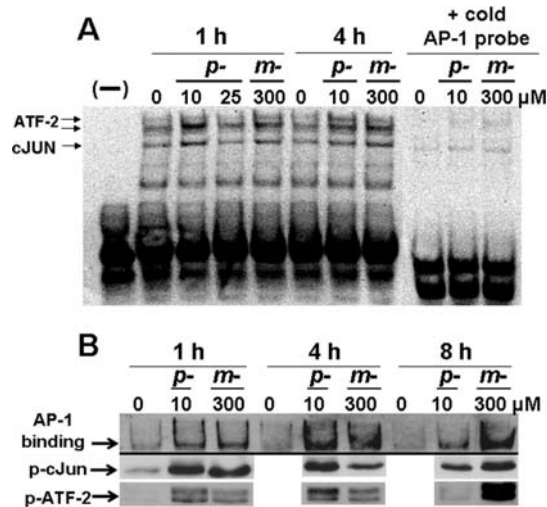


Figure 5. Effects of NO-ASA on transcription factor binding to the AP-1 complex. HT-29 cells were treated with the indicated concentrations of each NO-ASA isomer for 1-8 h. (A) EMSA for AP-1 in nuclear extracts isolated from control or treated HT-29 cells. (B) Non-denaturing gels were probed for cJun binding to the AP-1 probe and these were compared to Western blots of phosphorylated cJun and phosphorylated ATF-2 expression in whole cell lysates. Representative EMSA and Western blots of at least three independent experiments are shown.

To assess the relationship between MAPKs and p21, we investigated the effect of inhibiting p38 and JNK on p21. When p38 was inhibited by SB202190, the induction of p21 in response to either one of the NO-ASA isomers was reduced (Fig. 7A). In contrast, SP600125, the inhibitor of JNK, enhanced the induction of p21 by *p*-NO-ASA and to a much lesser degree by *m*-NO-ASA. These findings were assessed further by knocking down the expression of p38 and JNK using siRNAs. As shown in Fig. 7B, when the expression of p38 was knocked down in BxPC-3 cells, both *m*- and *p*-NO-ASA failed to induce p21 (HT-29 cells are resistant to transfection with these siRNAs). Knocking-down the expression of JNK enhanced the expression of p21 in response to *p*-NO-ASA but the induction of p21 in response to *m*-NO-ASA was lost, indicating the opposite roles of JNK in signaling by these two isomers. Interestingly, the combined suppression of p38 and JNK by the respective siRNAs essentially eliminated the induction of p21 by either isomer (Fig. 7B).

Control experiments demonstrated that siRNA directed against p38 reduced p38 levels to 19% of those in response to non-specific siRNA. Also following the combined insertion of JNK1/2- and p38-directed siRNAs, the expression of total p38 was lower than 5% of the control. Similar results were obtained after transfection with siRNA directed against JNK1/2 or with both JNK1/2- and p38-directed siRNAs, where the expression of JNK1/2 was clearly reduced compared to controls, especially its lower band (data not shown).

Gene silencing of p21 or MAPKs blocks the suppression of cell growth by NO-ASA. BxPC-3 cells transfected with siRNA directed at p38 or JNK1/2 alone failed to alter the effect of *p*-NO-ASA on cancer cell growth (Fig. 8). However, silencing both JNK1/2 and p38, or p21, inhibited significantly the effect of *p*-NO-ASA on cell growth. In contrast, silencing of only p38, JNK1/2 or p21 reduced the effect of the *meta* isomer

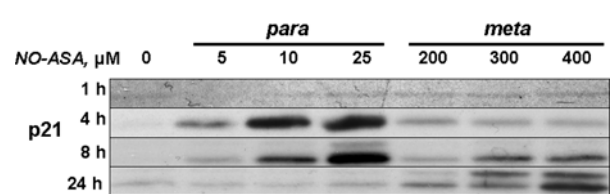


Figure 6. Effects of *para* and *meta* isomers of NO-ASA on p21 protein levels. HT-29 cells were treated with the indicated concentrations of each NO-ASA isomer for 1-24 h. Western blots for p21 are shown. For each time point, comparisons should be made against the control (0) which was electrophoretically separated on the same polyacrylamide gel and transferred to the same membrane as the treated lysates for the same incubation period. Representative Western blots of at least three independent experiments are shown.

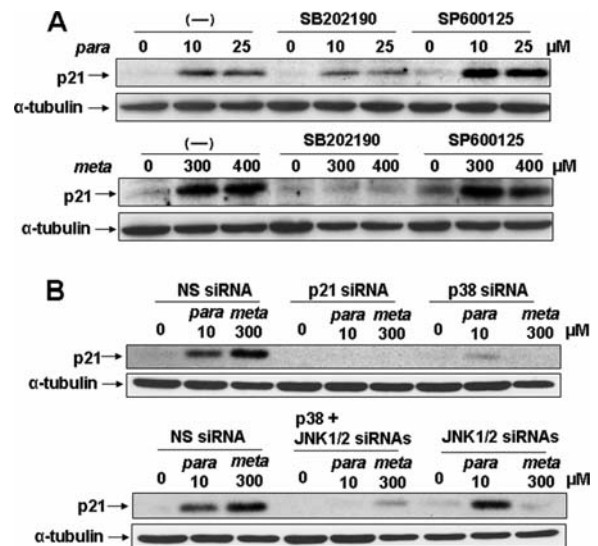


Figure 7. Effects of JNK and p38 inhibition on the induction of p21 expression. (A) HT-29 cells were treated with MAPK inhibitors SB202190 (p38) or SP600125 (JNK) both at 25 μ M for 30 min before further incubation with either NO-ASA isomer for 8 h. Western blots for p21 are shown. (B) BxPC-3 cells were transfected with siRNA directed against p21, p38 and/or JNK mRNA as described under Materials and methods before Western blotting. Western blots for p21 are shown. α -tubulin is shown as a loading control. Representative Western blots of at least three independent experiments are shown.

on cell growth, as did the combined silencing of p38 and JNK1/2 (Fig. 8). These results indicate that p21 is a pivotal target for both isomers. However, p38 and JNK1/2, both upstream of p21, play different roles in the effect of the two NO-ASA isomers on cell growth. Whereas both p38 and JNK1/2 are required for the effect of *p*-NO-ASA on cell growth; silencing either p38 or JNK1/2 alone is enough to rescue the growth inhibitory effect of the *meta* isomer.

The differential signaling events of the two positional isomers of NO-ASA. To summarize our findings on NO-ASA signaling, we have divided the effects of the two isomers into early (1-4 h) and late (8-24 h) effects. In addition, to provide an overall semiquantitative assessment of the effect of each isomer during these two time periods, we assigned an overall value according to the intensity of the effect. Fig. 9, illustrating what was detailed above, highlights the principal differences

Table I. Effects of *para* and *meta* isomers of NO-ASA on the cell cycle.

	Control	<i>p</i> -NO-ASA		<i>m</i> -NO-ASA	
		0.5 x IC ₅₀	1 x IC ₅₀	0.5 x IC ₅₀	1 x IC ₅₀
4 h					
G ₀ /G ₁	40.0	42.9	43.1	41.6	39.4
S	32.2	32.4	35.3	31.5	33.2
G ₂ /M	27.8	24.6	21.7	26.7	27.5
24 h					
G ₀ /G ₁	57.2	55.2	53.4	51.6	48.1
S	20.0	21.9	22.4	25.0	18.3
G ₂ /M	21.9	22.1	23.3	22.0	31.4

HT-29 cells were incubated for 4 or 24 h with 0.5 x or 1 x IC₅₀ of either the *para* or the *meta* isomer of NO-ASA. DNA content was determined from PI fluorescence as in Materials and methods. Percentages of the distribution of cells in G₀/G₁, S and G₂/M phases are shown. Values are representative of three independent experiments; results were within 10%.

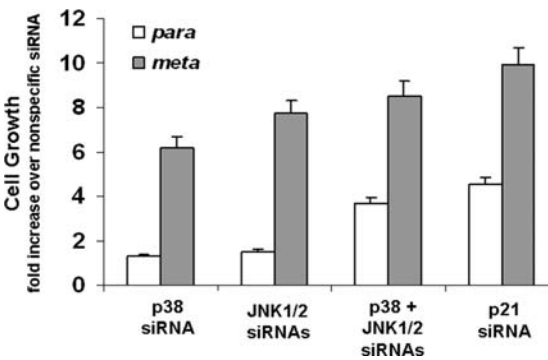


Figure 8. Effect of silencing JNK1/2, p38 or p21 on the growth inhibitory effect of NO-ASA. BxPC-3 pancreatic cancer cells were transfected with siRNA directed against p21, JNK1/2 and/or p38 mRNA. Forty-eight hours after transfection, cells were replated on 96-well plates and treated with either isomer of NO-ASA for 24 h before MTT assay was performed. Data are expressed as the fold increase over the cell growth of non-specific siRNA control for each treatment.

between the two isomers: a) *p*-NO-ASA exerts its effect earlier than *m*-NO-ASA; b) the predominant effect of *m*-NO-ASA is on ERK1/2 and Akt; whereas that of *p*-NO-ASA is on JNK1/2, while both activate p38, with *p*-NO-ASA showing a stronger and earlier effect; c) ATF-2 is more responsive to *m*-NO-ASA and c-Jun to *p*-NO-ASA; d) both isomers seem to have similar effects on AP-1 binding, the main difference between them being the timing of the effect; *p*-NO-ASA's effect is early and *m*-NO-ASA's is late; e) *p*-NO-ASA has an earlier and stronger effect on p21, while *m*-NO-ASA's effect occurs later and is weaker and f) cell cycle changes follow the effect on p21 expression.

Discussion

Isomerism can influence drug activity, at times profoundly (13-15). Our study, focused on the *m*- and *p*-positional isomers of NO-ASA, establishes a striking difference in biological activity between them. Their most important difference

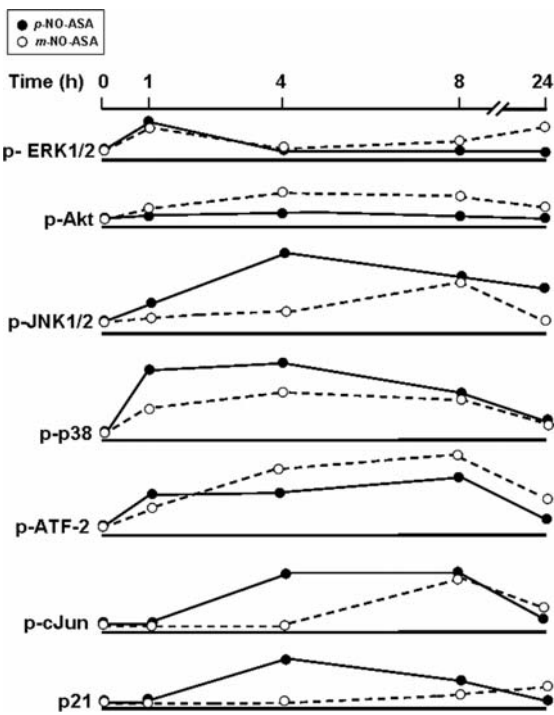


Figure 9. Scheme showing the differences in the effects on MAPK signaling triggered by either isomer of NO-ASA. The time response during which each change occurred and a rough representation of their relative intensity based on the quantification of the shown data. To note, the difference in the intensity and the time response for each particular protein activation or level measured is shown. The *para* NO-ASA (10 μ M) time responses for each parameter measured are shown in full lines with full dots, while the time responses of *meta* NO-ASA (300 μ M) are shown in broken lines with empty dots.

concerns their ability to inhibit colon cancer cell growth, with their IC₅₀ values differing by about 20-fold (*m*-NO-ASA = 230 \pm 15 μ M; *p*-NO-ASA = 12 \pm 5 μ M). The signaling studies that we performed provide at least a partial explanation of this dramatic difference in potency between the two positional isomers.

Both NO-ASA isomers differentially affected the three individual pathways of the MAPK signaling cascade and the Akt pathway. The Akt kinase participates in the inhibition of the intrinsic apoptotic pathway, by phosphorylating Bad, which prevents its translocation to the mitochondria. *p*-NO-ASA failed to activate Akt, as evidenced by the absence of change of the levels of its phosphorylated forms, while *m*-NO-ASA activated Akt, its effect peaking at 4 h. Although, the summation of the signaling effects of the *meta* isomer results in cell growth inhibition, the increased activation of Akt suggests an early anti-apoptotic response. However, this anti-apoptotic effect is overwhelmed by the later growth inhibitory events induced by *m*-NO-ASA.

NO-ASA isomers had little effect on ERK1/2 activation. The *para* isomer at its highest concentration activated ERK1/2 rapidly, while the *meta* isomer activated ERK1/2 modestly at 24 h. It is unlikely, however, that the ERK1/2 pathway mediates the growth inhibitory effect of NO-ASA, especially for *p*-NO-ASA, since we have previously shown that inhibition of ERK1/2 activation by the ERK1/2 inhibitor PD98059 had no effect on the ability of *p*-NO-ASA to inhibit cell growth (19).

p-NO-ASA activated JNK and p38 along with their respective downstream transcription factors, cJun and ATF-2. The activation of these two MAPKs was quantitatively pronounced; the phosphorylation of p38 increased within the first hour of treatment and continued to rise at least for 8 h at all concentrations (and for 24 h at its highest concentration). These changes were accompanied by changes in the levels of phosphorylated ATF-2, which also increased rapidly after *p*-NO-ASA treatment. On the other hand, *m*-NO-ASA increased p38 phosphorylation more gradually than the *para* isomer, peaking at 8 h. Moreover, the response to the *meta* isomer was weaker than to the *para*, leading to a later and modest increase in ATF-2 phosphorylation.

The phosphorylation of both isoforms of JNK, JNK1 and JNK2, increased rapidly in response to *p*-NO-ASA, reaching essentially maximal levels by 1 h. Of note, treatment with the *meta* isomer, led to a delayed and weaker induction of phosphorylation of the JNK isoforms, peaking after 8 h of treatment. Activation of phosphorylated cJun by the activated JNK was evident after 8 h for both NO-ASA isomers. Whereas *p*-NO-ASA increased cJun phosphorylation strongly and progressively during the first 8 h, the analogous effect of the *meta* isomer was only modest in intensity. This finding is in agreement with the degree of JNK1/2 phosphorylation that each isomer induced.

p21, a key signaling downstream molecule, was activated in response to MAPK activation, ultimately leading to cell cycle deceleration and cell growth inhibition. The differences in the time course for JNK and p38 MAPK activation by either NO-ASA isomer, were reflected in the induction of p21 protein expression. The expression of the cyclin-dependent kinase inhibitor p21, which blocks cell cycle progression by inhibiting the activity of cyclin/Cdk2 complexes (26-28), was differentially upregulated by the NO-ASA isomers. Whereas *p*-NO-ASA induced the rapid and strong expression of p21 in a dose- and time-dependent manner, the *m*-NO-ASA gradually induced a weaker p21 expression, peaking at 24 h-treatment. This differential effect was also evident in the way these

isomers arrested the cell cycle. While the *para* isomer blocked the cell cycle as early as 4 h, the *meta* isomer arrested the cell cycle later (detected at 24 h). The cell cycle effect by the NO-ASA isomers was clearly correlated with p21 levels.

The specific inhibition of JNK using a pharmacological inhibitor failed to block the effect of both NO-ASA isomers on p21 induction, but the inhibition of p38 did block the effect of either *p*- or *m*-NO-ASA on p21 induction. This finding suggested that the p38 signaling cascade mediates the effect of both NO-ASA isomers on p21 induction. This conclusion was, however, tentative because it was based on the use of pharmacological inhibitors, which always have the potential of effects beyond their target proteins. The studies using either siRNA for gene silencing were, therefore, needed for a thorough assessment of the contribution of p38 and JNK. Whereas transfections with siRNA directed at JNK1/2 alone failed to alter the effect of either *para* or *m*-NO-ASA on p21 induction, the silencing of p38 alone or the combined silencing of JNK1/2 and p38 inhibited significantly the effect of both NO-ASA isomers on p21 induction. This finding confirms that p38 MAPK mediates p21 induction by NO-ASA.

When we analyzed the effect of JNK and p38 pathway on cell growth, we observed that transfections with siRNA directed at p38 or JNK1/2 alone failed to alter the effect of *p*-NO-ASA on cancer cell growth. In contrast, the combined silencing of JNK1/2 and p38 inhibited significantly the effect of *p*-NO-ASA on cell growth. The silencing of p21 also blocked the cell growth inhibitory effect of both isomers. These findings, being in excellent agreement with the results obtained using pharmacological inhibitors, lead to the conclusion that *p*-NO-ASA needs to stimulate the p38 and JNK pathways to inhibit cell growth. On the contrary, *m*-NO-ASA needs to modulate either p38 or JNK to inhibit cell growth. p21 is a pivotal growth inhibitory target for both isomers.

The present structure-activity study underscores the complexity of the effects of NO-ASA isomers on cancer cell growth. We have previously shown that there is an excellent correlation between the initial rate of NO release by the different NO-ASA molecules (*meta* and *para*) and their cell inhibitory potencies (16). This finding emphasizes the contribution of NO to the biological effect of NO-ASA. However, regardless of these findings, the -ONO₂ group may not be the sole determinant of the biological activity of NO-ASA. Interestingly, the spacer is also important for NO-ASA's effect (29,30). It is conceivable that the two isomers generate different intermediates (quinone methide vs. a carbocation) leading to different biological responses (29). Furthermore, there are differences in the metabolism between the two isomers (22). These differences could contribute to the differential response of the cells to the two NO-ASA isomers. For example, both isomers of NO-ASA start with a common deacetylation step and end up with similar end products. Following cleavage of the ester bond between the two benzene rings, the *para* isomer release NO₃⁻ and form end products rapidly via a putative short-lived intermediate, whereas the *meta* isomer forms the stable (nitroxymethoxy) phenol, which releases NO₃⁻ and forms end products at a much slower rate (22). This may in part explain the different

time-profiles of the MAPK activation and p21 induction observed between the two isomers.

In conclusion, our study provides clear evidence that positional isomerism influences in an important way the NO-ASA molecule with regard to the inhibition of colon cancer cell growth. Our data also demonstrate that both NO-ASA isomers activate MAPKs, which regulate the expression of p21 leading to the growth inhibitory effect of NO-ASA. However, there are differences between the two isomers in the timing and intensity of these 'downstream' effects and they correlate with the growth inhibitory effect of NO-ASA. Our findings have potentially important implications for understanding the mechanism of action of these and related chemoprevention agents (31) as well as for the design of chemoprevention approaches.

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