

# Nitric Oxide-Donating Aspirin Inhibits Colon Cancer Cell Growth via Mitogen-Activated Protein Kinase Activation

Thomas R. Hundley and Basil Rigas

*Division of Cancer Prevention, Department of Medicine, State University of New York, Stony Brook, New York*

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## ABSTRACT

Nitric oxide-donating aspirin (NO-aspirin), representing a new concept in the development of more efficacious nonsteroidal anti-inflammatory drugs, consists of traditional aspirin bearing  $-ONO_2$ , which releases NO. Conventional aspirin prevents human colon cancer, but its toxicity precludes its application as a chemopreventive agent. NO-aspirin seems safer and in cultured cancer cells it is  $>1000$ -fold more potent than aspirin. To determine the mechanism by which NO-aspirin inhibits cell growth, we studied its effect on mitogen-activated protein kinase (MAPK) signaling in HT-29 human colon cancer cells. NO-aspirin stimulated the phosphorylation of extracellular signal-regulated kinase 1/2 and Akt only marginally. The greatest increases in phosphorylation were seen in cJun NH<sub>2</sub>-terminal kinase (JNK) and p38 MAP kinases, which were observed as

early as 5 min and after 1 h of treatment, averaged more than 10-fold over control. The activation of JNK and p38 was accompanied by large increases in the phosphorylation of the downstream transcription factors cJun and activating transcription factor 2 (ATF-2). We used specific MAPK inhibitors, small interfering (siRNA) gene silencing methods, and dominant-negative cJun to determine the relevance of these phosphorylation events to the ability of NO-aspirin to inhibit colon cancer cell growth. Only the dual inhibitor of p38 and JNK and the use of combined siRNA silencing of p38 and cJun abrogated the ability of NO-aspirin to block cell growth. Our data indicate that NO-aspirin is dependent on both the p38 and the JNK MAP kinase pathways for its ability to inhibit the growth of colon cancer cells.

The chemopreventive effect of aspirin against colon cancer has sparked interest in nonsteroidal anti-inflammatory drugs (NSAIDs) (Baron, 2003). Because of their limited efficacy and side effects, however, they have limited potential for cancer chemoprevention. The novel NO-donating nonsteroidal anti-inflammatory drugs hold the promise of overcoming the limitations of conventional nonsteroidal anti-inflammatory drugs (Rigas and Kashfi, 2004). They consist of a traditional NSAID to which a NO-donating moiety ( $-ONO_2$ ) is attached covalently via a spacer. NO-aspirin seems to be the most potent among them for the prevention of colon and several other cancers (Kashfi et al., 2002; Yeh et al., 2004). Its safety has been documented, at least preliminarily, in humans (Fiorucci et al., 2003). Both in vitro and in vivo work indicates that NO-aspirin potently inhibits colorectal carci-

nogenesis. For example, NO-aspirin inhibits the growth of human colon cancer cell lines, with its IC<sub>50</sub> for cell growth being over 1000-fold lower than that of aspirin (Yeh et al., 2004). In animal models, NO-aspirin inhibited gastrointestinal carcinogenesis more potently than aspirin (Bak et al., 1998; Williams et al., 2004; Kashfi et al., 2005). These findings have generated the important question concerning the mechanism underlying the extraordinary enhancement in potency displayed by NO-aspirin.

The mitogen-activated protein kinases (MAPKs) are a family of kinases that, in response to a variety of stimuli, transduce signals from the cell membrane to the nucleus, modulating gene transcription that leads to biological response (Pearson et al., 2001; Bode and Dong, 2004). MAPKs required for specialized cell functions controlling cell proliferation, cell differentiation, and cell death are deregulated in several malignancies, including colon cancer, and are presumed to be involved in their pathogenesis. Furthermore, recent data suggest that antineoplastic compounds modulate this pathway and such effects may mediate, at least in part, their

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**ABBREVIATIONS:** NSAID, nonsteroidal anti-inflammatory drug; NO, nitric oxide; NO-aspirin, nitric oxide-donating aspirin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, cJun NH<sub>2</sub>-terminal kinase; AP-1, activator protein 1; ATF-2, activating transcription factor 2; MAP, mitogen-activated protein; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) 1H-imidazole, HCl; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole; PD98059, 2'-amino-3'-methoxyflavone; SP600125, anthra[1,9-*cd*]pyrazol-6(2H)-one; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; p-, phosphorylated; siRNA, small interfering RNA; DN, dominant-negative.

pharmacological activity (Bode and Dong, 2004). The MAPK cascade, through sequential phosphorylations, diverges into selective activation of terminal kinases. There are three major groups of distinctly regulated MAPK cascades: ERK1/2, JNK, and p38 MAPK.

The two ERK isoforms, ERK1 and ERK2, target not only transcription factors but also membrane proteins (phospholipase A<sub>2</sub>) and cytoplasmic kinases. How they affect cell function is not precisely known, but potentially important effects include the ERK1/2-dependent regulation of the AP-1 transcription factors and the phosphorylation of the Elk-1 by ERK1/2. The potential involvement of the ERK1/2 pathways in carcinogenesis is underscored by their enhanced activation in a variety of tumors (Platanias, 2003; Wada and Penninger, 2004), including colon cancer (Sun and Sinicrope, 2005). These cascades are likely involved in tumor cell survival and/or proliferation.

The JNK pathway, activated by chemical and radiation-induced stresses and by inflammatory cytokines, is involved in the regulation of cell proliferation and apoptosis (Manning and Davis, 2003). cJun and ATF-2 are substrates of its three isoforms, JNK-1, JNK-2, and JNK-3. Of the upstream JNK activators, mitogen-activated protein kinase kinase 4 is activated by environmental stress, whereas mitogen-activated protein kinase kinase 7 is activated by cytokines. Of the four known negative JNK regulators, nitric oxide is most relevant to the present work. JNK pathways show an interesting dichotomy, having displayed both an oncogenic and a proapoptotic function. In the former context, the JNK cascade seems to initiate cellular transformation, whereas its proapoptotic function seems to be required for the apoptosis induced by chemotherapeutic agents.

The p38 MAPK pathway (Engelberg, 2004), similar to some extent to the other two pathways, is involved in inflammation, cell growth, cell death, and cell differentiation. Activated by a similar array of stimuli, including proinflammatory mediators, growth factors, and environmental factors, it has a complex role in cancer, being, for example, able to both promote and suppress proliferation of leukemia cells.

Although MAPKs function, in general, as autonomous signaling modules, there is at times significant cross-talk between them. A not unusual result of such cross-talk is the simultaneous activation of two pathways, as is the case with p38 MAP kinase and the JNK pathway. The cellular context determines the outcome of such cross-talk.

The profound cell kinetic effect of NO-aspirin, encompassing cell proliferation, cell cycle, and cell death, combined with the role of MAPK cascades in these cellular events that are critical to carcinogenesis, prompted us to examine whether NO-aspirin acts, at least in part, by modulating this extensive signal transduction system. Our findings demonstrate that NO-aspirin indeed modulates these cascades and that its particular effect on the JNK signaling pathway is a pivotal event in its remarkable pharmacological action.

## Materials and Methods

**Materials.** NO-aspirin and its structural analogs (Kashfi et al., 2005) were as follows: *para* isomer [NCX-4040, 2-(acetyloxy) benzoic acid 4-(nitrooxy methyl) phenyl ester], *meta* isomer (NCX-4016, 2-(acetyloxy) benzoic acid 3-(nitrooxy methyl) phenyl ester], and denitrated NO-aspirin [2-(acetyloxy)benzoic acid 4-(hydroxymethyl-

phenyl ester)]; they were gifts from Nicox S.A. (Sophia Antipolis, France). Antibodies against cJun, ATF-2, JNK, p38, ERK1/2, and Akt were from Cell Signaling Technology Inc. (Beverly, MA). Horseradish peroxidase-conjugated antibodies against  $\alpha$ -tubulin, mouse IgG, or rabbit IgG were from Calbiochem (San Diego, CA). MAPK inhibitors SB202190, SB203580, and PD98059, and wortmannin were from Calbiochem. The SP600125 MAPK inhibitor was from Alexis Biochemicals (Lausanne, Switzerland). Cell lines were from American Type Culture Collection (Manassas, VA), and cell culture reagents were from Mediatech (Herndon, VA).

**Western Blots.** Electrophoresis of cell lysates were performed on 10% SDS-polyacrylamide gel electrophoresis gels as described previously (Hundley et al., 2004); protein transfers were onto nitrocellulose membranes, which were stripped and reprobed when needed.

**Cell Culture and Treatments.** Cells were grown to 80% confluence in either McCoy's or RPMI 1640 medium (with 10% fetal calf serum, 1000 U/ml penicillin, and 1000  $\mu$ g/ml streptomycin). For treatment with various reagents,  $1.0 \times 10^6$  cells were plated in wells on six-well plates (Corning Glassworks, Corning, NY). NO-aspirin and other reagents were incubated with cell monolayers in the presence of 1% dimethyl sulfoxide in culture medium. After incubation, cultures were placed on ice, the medium was collected, and cells were washed twice with phosphate-buffered saline. Lysis buffer (20 mM HEPES, 50 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.3, with 10% glycerol, 1% Triton X-100, 20  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2.5 mM 4-nitrophenylphosphate) was added to cultures in wells. The floating cells from the cultures were pooled with the adherent cell lysates. Cell proliferation was assayed using the MTT colorimetric assay kit (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical, Rockford, IL).

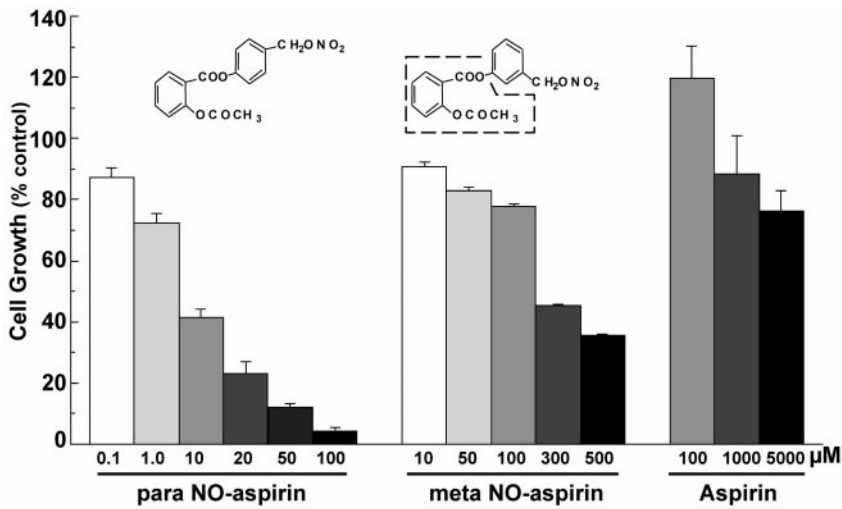
**Cell Transfections.**  $-/-$ cJun in the TAM-67 expression vector was the kind gift of Dr. Z. Dong, Hormel Institute, Austin, MN (Huang et al., 1999). HT-29 cells were transfected with plasmid DNA 2  $\mu$ g/well using Lipofectamine 2000 following the manufacturer's protocol (Invitrogen, Carlsbad, CA) and selected with G418 (Geneticin) at 600  $\mu$ g/ml.

**Statistical Methods.** Continuous outcomes were compared among drug concentrations and, alternatively, between drugs at specified concentrations by using analysis of variance followed by Tukey's method for multiple pairwise comparisons (Miller, 1981). All tests were considered statistically significant at  $P < 0.05$ .

## Results

**NO-Aspirin Inhibits HT-29 Colon Cancer Cell Proliferation.** Initially, we evaluated in our cell culture system two positional isomers of NO-aspirin, the *meta* and *para* isomer, denoting the position of the -CH<sub>2</sub>ONO<sub>2</sub> group in the benzene ring with respect to the ester bond linking it to the conventional aspirin moiety. HT-29 colon cancer cells were treated either with one of these NO-aspirin positional isomers or with aspirin, and their growth was determined 24 h later. As seen in Fig. 1, all three compounds inhibited the growth of these cells, but their IC<sub>50</sub> values differed greatly: *m-* =  $316 \pm 12$   $\mu$ M (mean  $\pm$  S.E.M., for this and all subsequent values), *p-* =  $7.0 \pm 3.0$   $\mu$ M, and aspirin =  $>5000$   $\mu$ M. Thus, both NO-aspirin isomers showed much enhanced potency compared with aspirin. However, the *para* isomer of NO-aspirin was the most potent; thus, our subsequent work was focused on the *para* isomer.

**NO-Aspirin Activates ERK1/2 and Akt MAP Kinases.** To explore the signaling pathways used by NO-aspirin to mediate its cell growth inhibitory effect, we examined MAP kinases thought to modulate cell kinetics (proliferation and cell death). ERK1/2 is known to regulate cell growth and

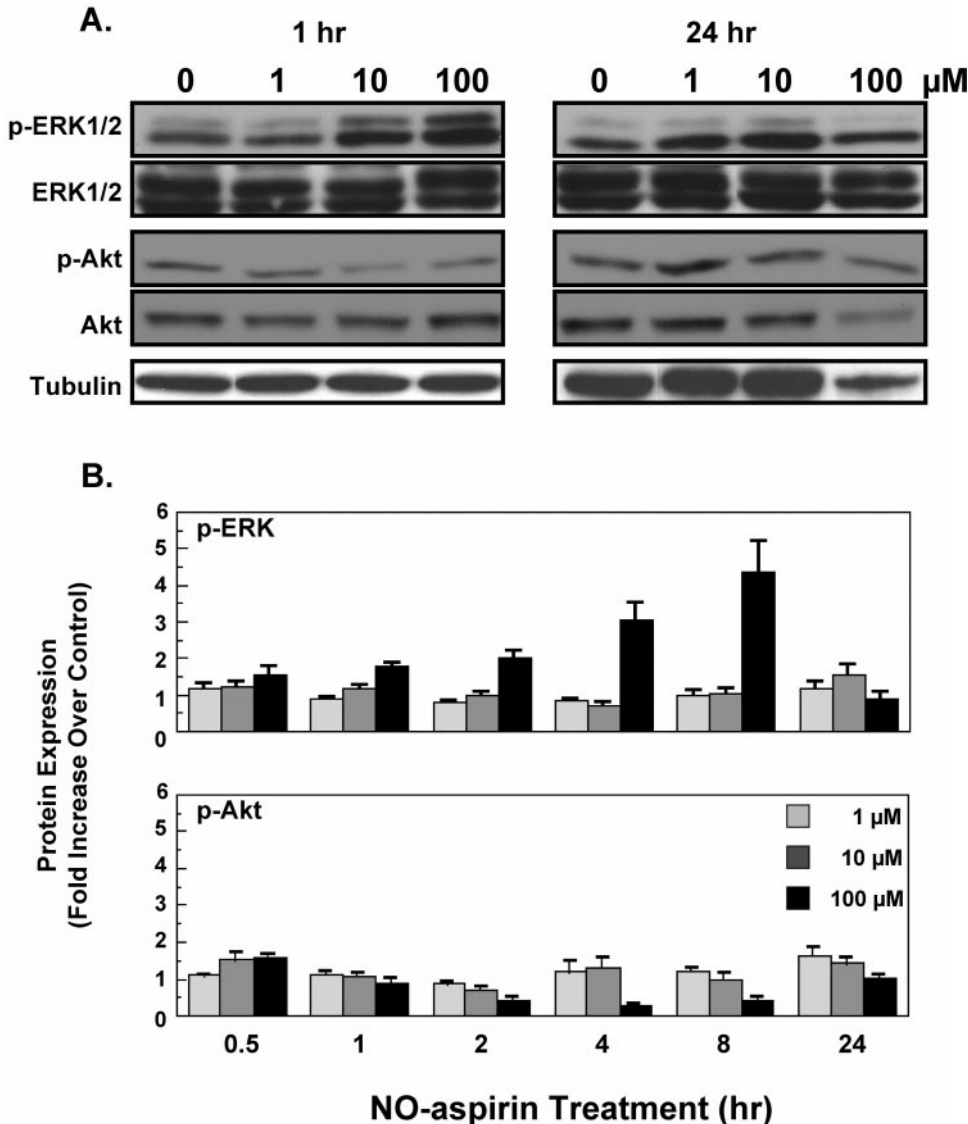


**Fig. 1.** Effects of NO-aspirin and aspirin on cell growth. HT-29 colon cancer cell cultures were treated with various concentrations of *para* or *meta* NO-aspirin or aspirin for 24 h. Cell growth was assayed using the MTT cell proliferation assay. Inset shows the structures of *para* and *meta* NO-aspirin, with conventional aspirin within the box. Data are expressed as percentage of cell growth observed in untreated cultures. The results are the average of more than three experiments, and bars represent the S.E.M.

differentiation, whereas Akt is involved in apoptotic signaling (Testa and Bellacosa, 2001).

To establish an initial relationship between changes in the levels of activation of the various MAP kinases and the inhi-

tion of cell proliferation produced by treatment with *para* NO-aspirin, lysates from HT-29 cell cultures treated for up to 24 h were analyzed by Western blotting for the phosphorylated forms of selected MAP kinases (Fig. 2).



**Fig. 2.** Activation of MAPK by NO-aspirin. HT-29 or SW-480 cells were incubated with NO-aspirin for up to 24 h before cultures were lysed, and the expression of proteins was determined by Western blotting. A, representative Western blots of HT-29 cell lysates probed for p-ERK1/2, p-Akt, total ERK1/2, total Akt, and  $\alpha$ -tubulin. B, graphic representation of averages of protein expression in HT-29 cell lysates. Concentrations of NO-aspirin for each time point are 1, 10, or 100  $\mu$ M. Similar results were observed with SW-480 cell cultures. Calculations were determined as the fold increase over control band density minus background density. The results are the average of more than three experiments, and error bars represent the S.E.M.

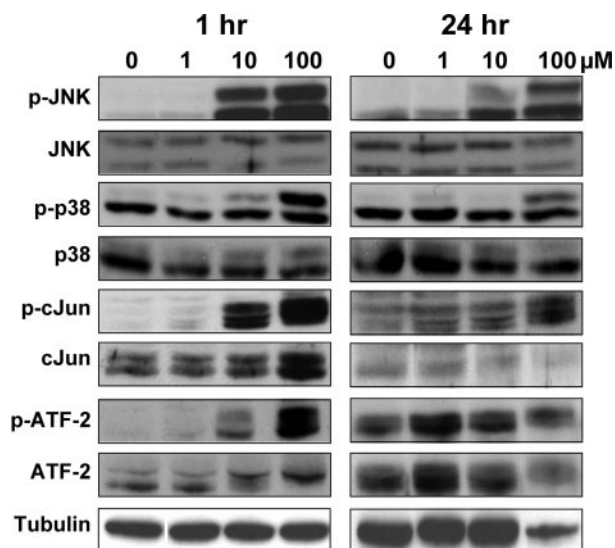


Both ERK1/2 and Akt exhibited basal levels of phosphorylated proteins. The levels of phosphorylated ERK2 in response to 1 and 10  $\mu\text{M}$  NO-aspirin fluctuate modestly over the 24-h observation period; however, there are no significant changes in its concentration. In contrast, significant increases in the phosphorylation of ERK2 are seen after 2 h of treatment with 100  $\mu\text{M}$  NO-aspirin, reaching their maximum at 8 h (4.5-fold over baseline), returning to normal by 24 h. The phosphorylation of ERK1 followed an almost identical pattern of change as ERK2 (data not shown in the graph; Fig. 2B).

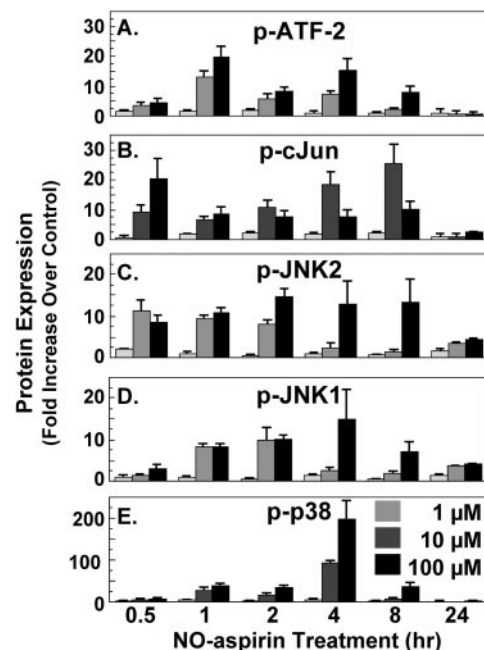
The phosphorylation of Akt followed a similar pattern to ERK1/2 for NO-aspirin (1–10  $\mu\text{M}$ ); phosphorylation levels also fluctuating modestly without a statistically significant change from baseline. In contrast to ERK1/2, at 100  $\mu\text{M}$  NO-aspirin, the levels of phosphorylated Akt decrease progressively starting at 1 h and reach their nadir at 4 h (27% baseline), after which there is a progressive increase returning toward baseline values at 24 h.

**NO-Aspirin Activates JNK and p38 MAP Kinases.** In unstimulated HT-29 cells, phosphorylated JNK was essentially undetectable (Fig. 3). Within 30 min after treatment of these cells with NO-aspirin, two very strong bands of phosphorylated JNK appeared (Fig. 3); we determined the expression of these two bands individually. Occasionally, we detected a third band of higher molecular mass (>54 kDa), likely resulting from hyperphosphorylation of JNK1/2 (data not shown).

There is a clear time- and concentration-dependent increase in phosphorylated JNK (Fig. 4A) by NO-aspirin. NO-aspirin (1  $\mu\text{M}$ ) failed to stimulate significant formation of p-JNK. In contrast, NO-aspirin at 10 and 100  $\mu\text{M}$  concentrations greatly increased the formation p-JNK, but with different kinetics. Thus, at 10  $\mu\text{M}$ , the upper band appears at 30 min (10-fold over baseline), and the lower band reaches that level at 1 h; both peak at 2 h returning to about 3-fold at 24 h. NO-aspirin (100  $\mu\text{M}$ ) stimulated a similar response to both



**Fig. 3.** Activation of MAPK by NO-aspirin. HT-29 or SW-480 cells were incubated with 1, 10, or 100  $\mu\text{M}$  NO-aspirin for up to 24 h before cultures were lysed, and the expression of proteins was observed by Western blotting. Shown are representative Western blots of HT-29 cell lysates probed for p-JNK, p-p38, p-cJun, p-ATF-2, and the total expressions of JNK, p38, cJun, ATF-2, and  $\alpha$ -tubulin.



**Fig. 4.** Activation of MAPK by NO-aspirin. Graphic representation of protein expression in HT-29 cell lysates. HT-29 cells were incubated with NO-aspirin for up to 24 h before cultures were lysed, and the expression of proteins was observed by Western blotting. Concentrations of NO-aspirin for each time point are 1, 10, or 100  $\mu\text{M}$ . A, p-ATF-2. B, p-cJun. C, p-JNK2. D, p-JNK1. E, p-p38 (note compressed scale). Calculations determined as the fold increase over control band density minus background density. Results are the mean  $\pm$  S.E.M. of more than three experiments.

bands with the difference from the 10  $\mu\text{M}$  concentration being that a substantial decline in their levels begins at 8 h. Levels do not return to normal even at 24 h, remaining 5-fold greater than baseline. Of note, the total JNK protein did not change in response to NO-aspirin, with only its phosphorylation status being affected.

NO-aspirin stimulated the phosphorylation of p38 in a similar manner to JNK. The lowest concentration (1  $\mu\text{M}$ ) had no appreciable effect, whereas higher concentrations did stimulate the formation of phosphorylated p38 markedly. Appreciable induction of phosphorylated p38 was evident even at 30 min, and it reached its maximum at 4 h: 100- and 200-fold over baseline for 10 and 100  $\mu\text{M}$ , respectively. A rapid decline was evident at 8 h, and this dramatic effect had dissipated at 24 h. It is interesting to note that, as shown in Fig. 3, NO-aspirin induces a second band with slower electrophoretic mobility than phosphorylated p38 that was detected inconsistently.

**NO-Aspirin Activates AP-1 Complex Transcription Factors.** JNK and p38 MAP kinase signaling leads to gene activation via the activation of transcription factors of the AP-1 complex. JNK MAP kinase activates the transcription factors cJun and ATF-2, whereas p38 MAP kinase activates ATF-2. We examined whether NO-aspirin also activates and/or induces two transcription factors of the AP-1 complex, cJun and ATF-2 (Figs. 3 and 4).

Phosphorylation of cJun in response to NO-aspirin treatment of HT-29 cells is both time- and concentration-dependent. At the lowest concentration of NO-aspirin (1  $\mu\text{M}$ ), phosphorylation of cJun becomes clearly detectable at 1 h, remaining around the same low (but always significantly

above the baseline value) levels throughout the 24 h of observation. When the cells were treated with 10 or 100  $\mu\text{M}$  NO-aspirin, phosphorylated cJun was abundant as early as 30 min later, 10- and 20-fold higher than baseline, respectively. At 10  $\mu\text{M}$ , the levels of phosphorylated cJun rise steadily to a maximum of about 25-fold at 8 h, returning to nearly baseline values at 24 h. In contrast, at 100  $\mu\text{M}$  there is a rapid rise at 30 min, followed by a plateau at about 7-fold over baseline between 1 and 8 h, returning to about 2-fold over baseline values at 24 h.

Levels of phosphorylated ATF-2, detectable in untreated HT-29 cells, do not change appreciably in response to treatment with NO-aspirin (1  $\mu\text{M}$ ) over the period of observation. NO-aspirin (10  $\mu\text{M}$ ) increases the levels of phosphorylated ATF-2; maximal increase is noted at 1 h with a progressive decline to near baseline levels by 8 h. NO-aspirin (100  $\mu\text{M}$ ) also leads to maximal increase in the levels of phosphorylated ATF-2 at 1 h. Following this, levels drop to about 7-fold over baseline at 2 h and then increase to about 15-fold at 4 h, dropping to baseline by 24 h.

**NO-Aspirin Does Not Inhibit Phosphatase Activity.** One potential explanation for the increased levels of phosphorylated proteins that we observed in response to NO-aspirin treatment is that NO-aspirin inhibits phosphatase activity(ies), thus increasing their levels. To evaluate this possibility, we prepared protein lysates from NO-aspirin-treated cultures with and without phosphatase inhibitors. Lysates of HT-29 cells were collected in the presence or absence of  $\text{Na}_3\text{VO}_4$  and 4-nitrophenyl-phosphate (both phosphatase inhibitors). As shown in Fig. 5, in the absence of phosphatase inhibitors, all six proteins that we studied were dephosphorylated. This was clear-cut in the case of cells treated with NO-aspirin at either 1 or 10  $\mu\text{M}$ . However, some residual phosphorylation remained even in the absence of phosphatase inhibitors in cells treated with NO-aspirin at 100  $\mu\text{M}$ . The reasons for this are not readily apparent. Notwithstanding, our findings indicate that, overall, NO-aspirin stimulated phosphorylation rather than inactivated their phosphatases.

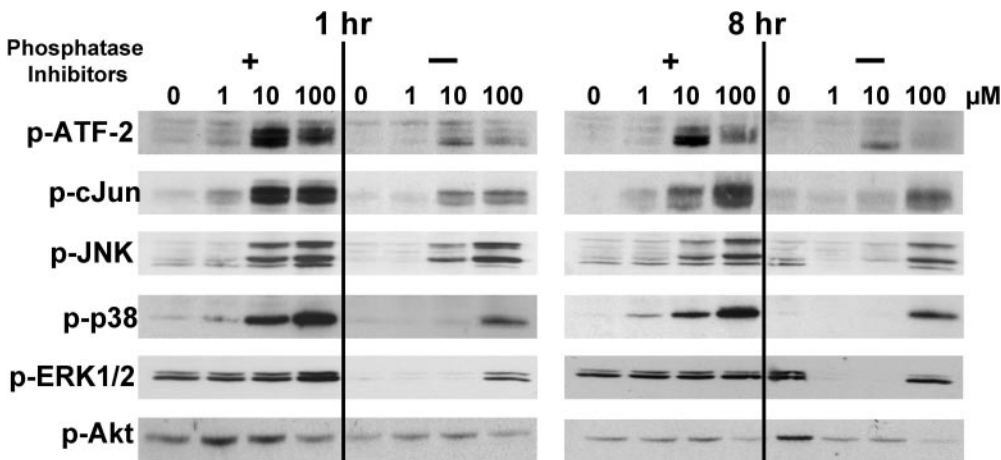
**Activation of MAP Kinases Correlates with Cell Growth Inhibition.** In an effort to assess the biological significance of the activation of MAP kinases that we observed, we ascertained whether inhibition of cell growth parallels MAP kinase activation. To this end, we used an analog of NO-aspirin that does not bare the  $-\text{ONO}_2$  moiety and is

known not to inhibit cell growth substantially. Additionally, we used traditional aspirin, which even at high concentrations has only a modest effect on cell growth.

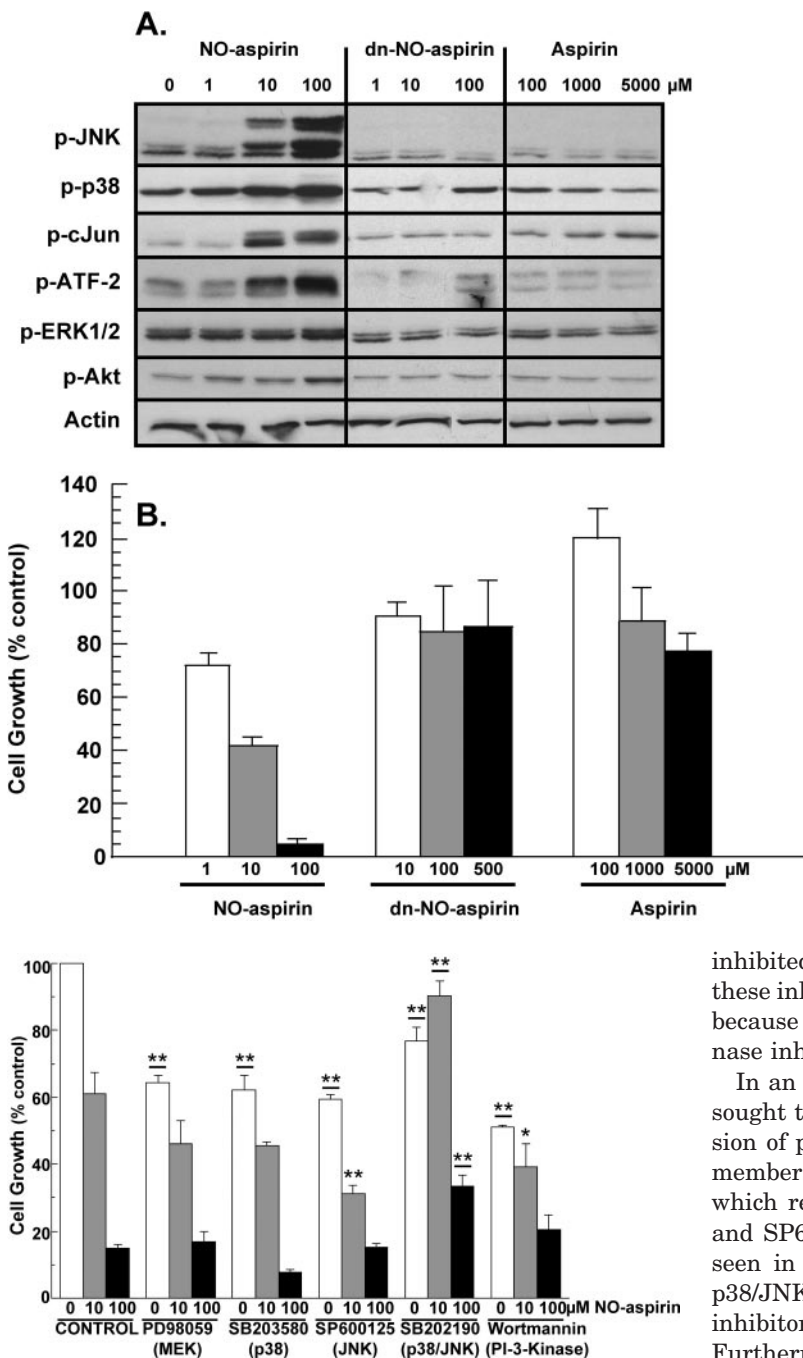
As shown in Fig. 6, NO-aspirin studied at 1 to 100  $\mu\text{M}$  inhibited HT-29 cell growth ( $\text{IC}_{50} = 7.0 \pm 3.0 \mu\text{M}$ ). This effect was accompanied by concentration-dependent activation of MAP kinases, as already described; a modest activation of Akt was also noted. In contrast, both the denitrated derivative of NO-aspirin (10–500  $\mu\text{M}$ ) and aspirin (100–5000  $\mu\text{M}$ ) inhibited cell growth only modestly, never reaching  $\text{IC}_{50}$  concentrations. Neither compound activated any of the six kinases that we studied. Although such correlational data do not necessarily prove that these kinases mediate the cell growth inhibitory effect of NO-aspirin, our finding is nevertheless consistent with this notion.

**Combined Inhibition of JNK and p38 Partially Blocks Cell Growth Inhibition by NO-Aspirin.** We sought to assess the biological significance of the activation of MAP kinases in the inhibition of HT-29 growth by NO-aspirin. To this end, we used five known pharmacological inhibitors of various MAP kinases. We used inhibitors of the activation of ERK1/2 (PD98059), p38 (SB203580), JNK (SP600125), and p38/JNK (SB202190) and of the Akt/phosphoinositide 3-kinase 3 pathway (wortmannin). HT-29 cells were treated with each of these inhibitors (each at 25  $\mu\text{M}$ , except for wortmannin at 100 nM) starting 30 min before the addition of NO-aspirin to the culture media; we used a low and a high concentration of NO-aspirin (10 and 100  $\mu\text{M}$ , respectively). Cell growth was determined at 24 h, and kinase expression was determined at 60 min.

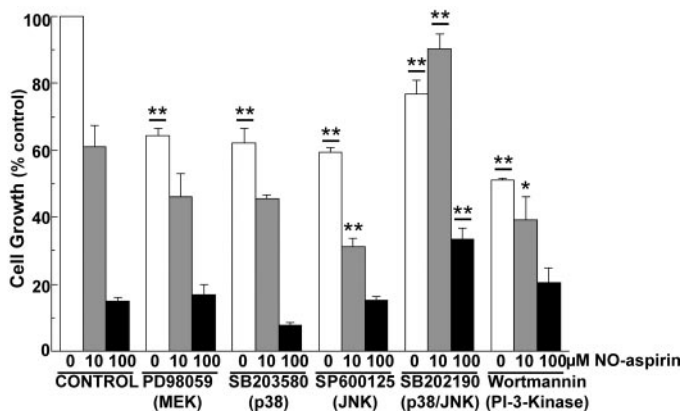
Of the five inhibitors, only SB202190 abrogated the inhibitory effect of NO-aspirin on cell growth; all others failed to substantially modify the growth inhibitor effect of NO-aspirin. As shown in Fig. 7, in the absence of NO-aspirin, SB202190 inhibited cell growth by 22% compared with control. At 10  $\mu\text{M}$  NO-aspirin, cell growth was inhibited by 40% and pretreatment with SB202190 completely reversed this effect, restoring cell growth to that of untreated control cells. At 100  $\mu\text{M}$  NO-aspirin, cell growth was inhibited by about 85%. SB202190 partially reversed this inhibitory effect, bringing cell growth to 35% of that of untreated cells; in other words, there were twice as many HT-29 cells when they were pretreated with the inhibitor. It is apparent that, at the higher NO-aspirin concentration, the cells are not fully re-



**Fig. 5.** Loss of phosphoprotein expression in the absence of phosphatase inhibitors. HT-29 cell cultures treated with NO-aspirin for 1 or 8 h were lysed with buffer containing or without phosphatase inhibitors, and the expression of phosphorylated proteins was determined by Western blotting.



**Fig. 6.** Comparison of MAPK activation by NO-aspirin, denitrated NO-aspirin, or aspirin. A, HT-29 cells were incubated with drug for 1 h before cultures were lysed, and the expression of proteins was observed by Western blotting. B, HT-29 cell cultures were treated with drug for 24 h, and then cell growth was assayed using the MTT cell proliferation assay. Data are expressed as percentage of cell growth observed in untreated cultures. Results are the mean ± S.E.M. of more than three experiments. dn-NO-aspirin, denitrated NO-aspirin.



**Fig. 7.** Effect of various MAPK inhibitors on growth inhibitory effect of NO-aspirin. HT-29 cell cultures were incubated with inhibitors for 30 min before adding NO-aspirin to cultures, and incubation continued for 24 h. Inhibitors used were 25 μM PD98059 (inhibits mitogen-activated protein kinase kinase-ERK), 25 μM SB203580 (inhibits p38), 25 μM SP600125 (inhibits JNK1, JNK2, and JNK3), 25 μM SB202190 (inhibits p38/JNK), and 100 nM wortmannin (inhibits phosphoinositide 3-kinase). After 24 h, cell growth was assayed with the addition of MTT labeling solution for the MTT assay. Data are expressed as percentage of cell growth observed in untreated cultures. Results are the mean ± S.E.M. of more than three experiments. The target of each inhibitor is indicated in parentheses. Statistically significant differences from the corresponding control values are indicated by asterisks (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ); all other differences are not statistically significant.

sponsive to the inhibitor; their commitment to cell death may be limiting their responsiveness.

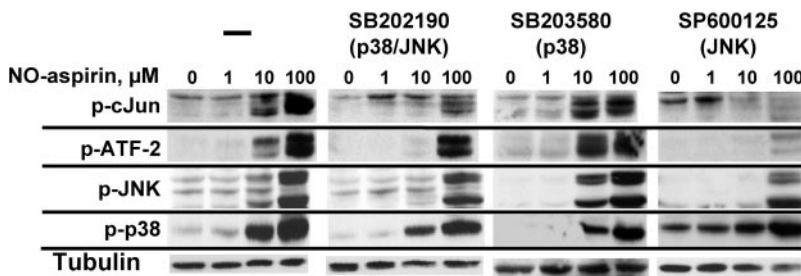
Regarding the other four inhibitors, each one of them alone

inhibited cell growth by about 40 to 50%. Attempts to use these inhibitors in combination gave uncertain results, likely because of greatly decreased cell proliferation by these kinase inhibitors in the absence of NO-aspirin.

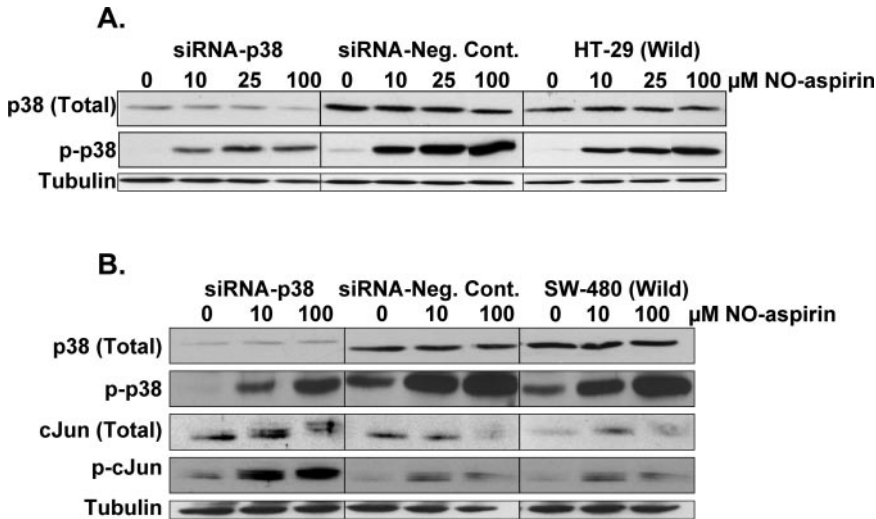
In an effort to understand the underlying mechanism, we sought to assess the effect of these inhibitors on the expression of phosphorylated cJun, ATF-2, JNK, and p38, all key members of the JNK/p38 pathway. We focused on SB202190, which reversed the effect of NO-aspirin, and on SB203580 and SP600125, which did not reverse the effect. As can be seen in Fig. 8, compared with the effect of the combined p38/JNK inhibitor on the four proteins, the effect of the p38 inhibitor is much weaker, especially at 10 μM NO-aspirin. Furthermore, after 24 h of treatment with NO-aspirin, the combined p38/JNK inhibitor was still effective in inhibiting NO-aspirin-stimulated phosphorylation of cJun and ATF-2, whereas the p38 inhibitor was no longer effective (data not shown). In contrast, and with the exception of phosphorylated p38, the inhibitory effect of the JNK inhibitor on these proteins is stronger than that of the combined p38/JNK inhibitor. Of note, the JNK inhibitor activated p38 in the absence of NO-aspirin or at 1 μM NO-aspirin; at higher NO-aspirin concentrations, however, it attenuated the effect of NO-aspirin on the activation of p38. The activation of p38 by SP600125 has been reported recently (Vaishnav et al., 2003).

**siRNA Silencing of Both p38 and cJun Reverses the Growth Suppression by NO-Aspirin.** Because of the previous results, we used another approach to ascertain the role of these MAPKs in mediating the growth inhibitory effect of





**Fig. 8.** Effect of MAPK inhibitors phosphoprotein expression. HT-29 cell cultures were incubated with inhibitors for 30 min before the addition of NO-aspirin to cultures, and incubation continued for 1 h. Inhibitors used were SB202190 (25  $\mu$ M), SB203580 (25  $\mu$ M), and SP600125 (25  $\mu$ M). After 1-h incubation with NO-aspirin, cultures were lysed and the expression of proteins was observed by Western blotting.

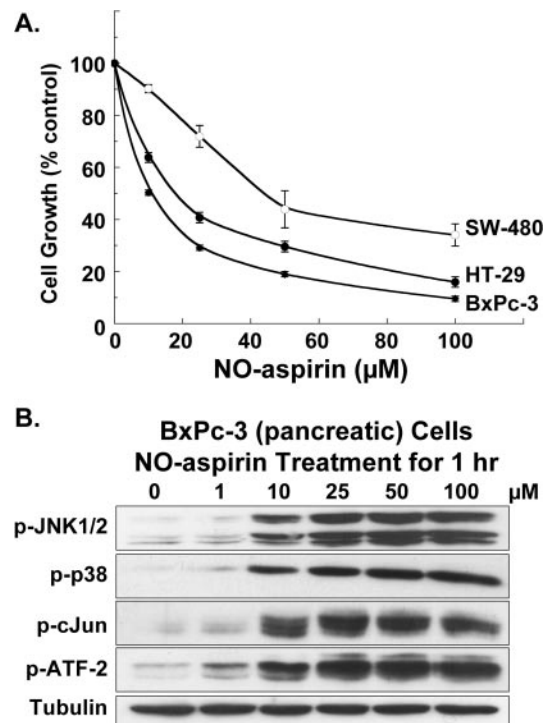


**Fig. 9.** Inhibition of p38 expression with siRNA in HT-29 and SW-480 cells. HT-29 (A) and SW-480 (B) cells were transfected for 48 h with siRNA directed against p38 mRNA as described under *Materials and Methods* before Western blotting. siRNA-treated and wild-type cells were treated with NO-aspirin (0, 10, 25, or 100  $\mu$ M).

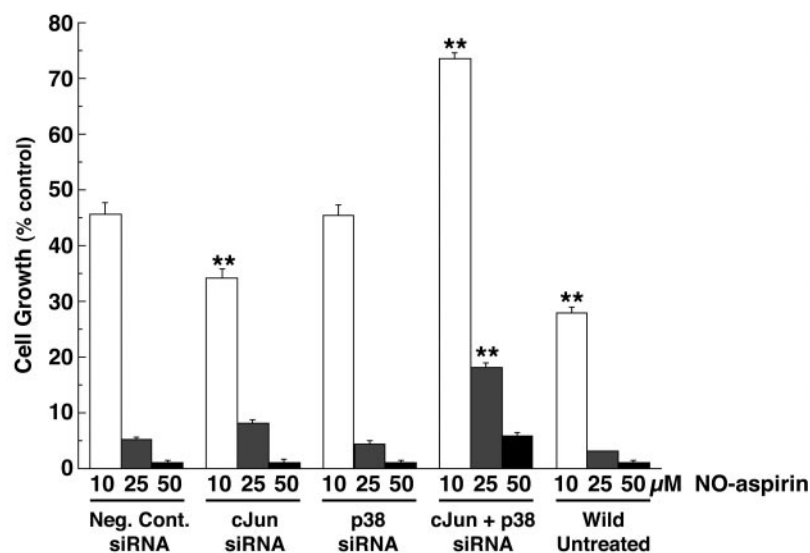
NO-aspirin. Since NO-aspirin elicits an early activation of both JNK and p38 MAPKs, and SB202190 is a dual p38/JNK inhibitor, we speculated that both the p38 and JNK pathways are important mediators of the effects of NO-aspirin.

To determine the role of p38 and JNK in NO-aspirin signaling, HT-29 and SW-480 cells were transfected with siRNA directed against the expression of either p38 or cJun, or JNK1 or JNK2. Forty-eight hours after transfection of cells with 100 nM siRNA directed against p38, the expression of total p38 in HT-29 and SW-480 cells was reduced, respectively, to 19 and 7% of the amounts expressed in control cultures (nonspecific siRNA) (Fig. 9). Despite these reductions in the expression of p38 there was no change in the  $IC_{50}$  for growth inhibition by NO-aspirin. Surprisingly, in the presence of NO-aspirin, the expression of both total cJun and phosphorylated cJun was increased (the latter more than the former) in both cell lines when transfected with siRNA directed against p38 (Fig. 9B). The possibility of a reciprocal regulation between the two proteins cannot be ruled out. The use of siRNA directed against JNK1, JNK2, or cJun was only partially successful, reducing the expression of these proteins in either HT-29 or SW480 cells at most by 50%.

In subsequent experiments, BxPc-3 (pancreatic carcinoma) cells were used for transfections of siRNAs directed at p38, cJun, JNK1, and JNK2; use of this cell line was necessitated because it was difficult to transfect HT-29 cells with these siRNAs. Similar to its effect on HT-29 and SW480, NO-aspirin also inhibited concentration dependently the growth of these cells (Fig. 10A). NO-aspirin activated JNK and p38 in a manner analogous to that observed in HT-29 cells (Fig. 10B). This effect was accompanied by phosphorylation of their dependent transcription factors cJun and ATF-2.



**Fig. 10.** Effect of NO-aspirin on growth and MAPKs in BxPC-3 pancreatic cancer cells. A, BxPC-3 pancreatic cancer cells and also HT-29 and SW480 colon cancer cells were treated with various concentrations of *para* NO-aspirin for 24 h. Cell growth was assayed using the MTT cell proliferation assay. Data are expressed as percentage of cell growth observed in untreated cultures. The results are the average of more than three experiments, and bars represent the S.E.M. B, representative Western blots of BxPc-3 cell lysates probed for p-JNK, p-p38, p-cJun, and p-ATF-2 expression;  $\alpha$ -tubulin was assayed as a loading control.



**Fig. 11.** Effect of siRNA inhibition of cJun and p38 expression in BxPc-3 cells on growth inhibitory effect of NO-aspirin. BxPc-3 pancreatic cancer cells were transfected with siRNA directed against cJun and/or p38 mRNA. Forty-eight hours after transfection, cells were replated on 96-well plates and treated with NO-aspirin for 24 h before MTT assay was performed. Data are expressed as percentage of cell growth of control. Statistically significant differences from the corresponding control values are indicated by asterisks (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ); all other differences are not statistically significant.

Transfections of BXPc-3 cells with siRNA directed at p38 or cJun alone failed to alter the effect of NO-aspirin on cancer cell growth. The combined insertion of cJun- and p38-directed siRNAs, however, inhibited significantly the effect on NO-aspirin on cell growth. As seen in Fig. 11, only when the expression of both cJun and p38 is attenuated in BxPc-3 cells is the effect of NO-aspirin on their growth reduced. Thus, these results support the observation seen with the combined pharmacological inhibition of these two pathways. Thus, based on these experiments it seems that both the JNK and the p38 MAPK pathways are required for the cell growth inhibitory effect of NO-aspirin.

To explore this mechanistic question further, we transfected HT-29 cells with a dominant-negative cJun (DNcJun) and assessed the ability of NO-aspirin to inhibit cell growth in its presence. Figure 12 shows typical results obtained with these experiments. Two of the transfectants were treated with various concentrations of NO-aspirin and compared with wild-type controls. The inhibitory effect on cell growth of the lowest concentration of NO-aspirin (10  $\mu$ M) was almost completely reversed by the DNcJun. This reversal was progressively less pronounced as the concentration of NO-aspirin increased. These findings indicate that cJun plays a potentially critical role in mediating the cell growth inhibitory effect of NO-aspirin, especially at its lower concentrations.

## Discussion

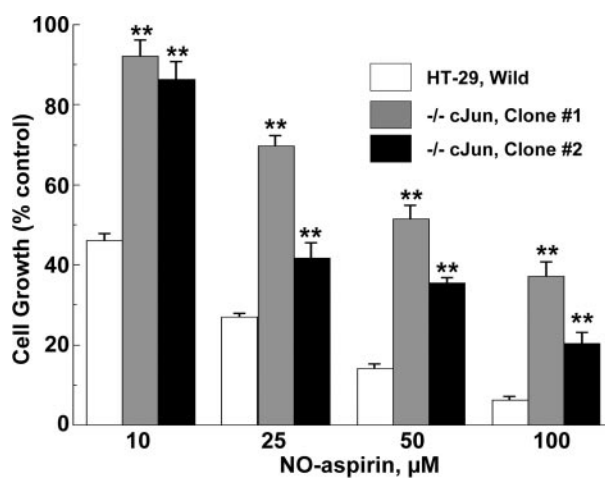
Our study confirms the extraordinarily enhanced growth inhibitory activity of NO-aspirin in colon cancer cells compared with traditional aspirin and indicates that it is mediated via NO-aspirin's effect on MAPK signaling pathways. Critical among them seems to be its combined activation by phosphorylation of p38 and cJun.

Traditional aspirin is a bona fide chemoprevention agent against colon cancer for which interventional studies have provided formal proof (Baron et al., 2003). The growth inhibitory effect of NO-aspirin is much stronger than that of aspirin, and this finding is in close agreement with reports from our laboratory (Williams et al., 2001; Kashfi et al., 2002) as well as those of others (Tesei et al., 2005). This enhanced effect of NO-aspirin is potentially very important and con-

ceptually lies at the heart of its anticancer effect. NO-aspirin's anticancer effect encompasses both cancer prevention and cancer treatment; the latter is evidenced by the effect of NO-aspirin on already formed intestinal tumors in *Min* mice (Kashfi et al., 2005). Importantly, NO-aspirin has already entered clinical trials for colon cancer prevention.

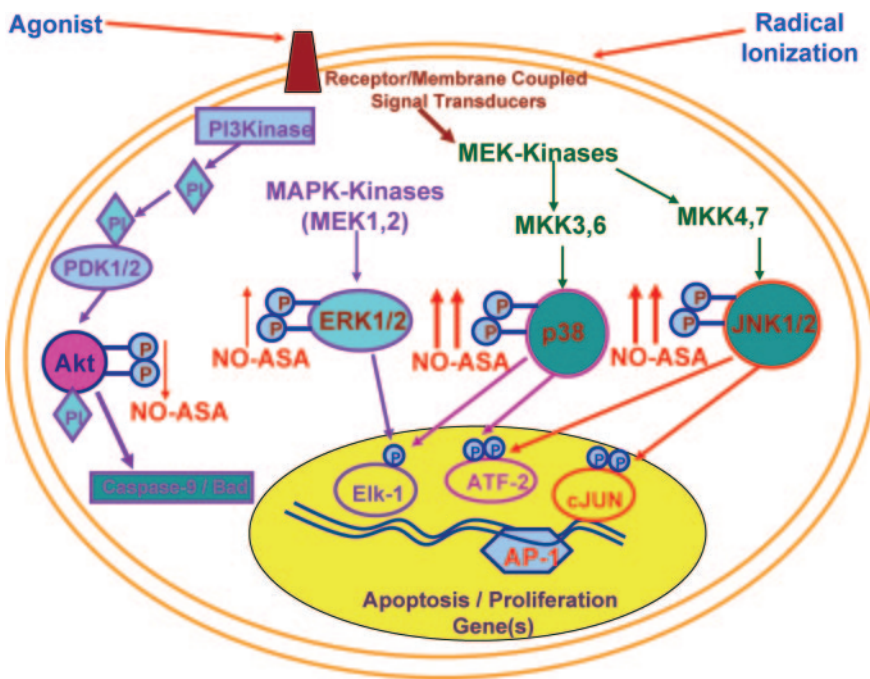
As summarized in Fig. 13, we studied the effect of NO-aspirin on three individual pathways of the MAPK signaling cascade and on the Akt pathway that is involved in the regulation of apoptosis. NO-aspirin failed to activate Akt or ERK1/2 as evidenced by the absence of change of the levels of their phosphorylated forms, with the exception of ERK1/2 that was elevated only by NO-aspirin (100  $\mu$ M) and only at 4 and 8 h.

It is unlikely that either pathway plays a role in mediating the growth inhibitory effect of NO-aspirin. Inhibition of ERK1/2 activation by pretreatment of cells with PD98059



**Fig. 12.** Effect of dominant-negative cJun on cell growth inhibitory effect of NO-aspirin. HT-29 cells were transfected to express  $-/-$ cJun (cannot be phosphorylated); after selection of colonies, cells were assayed for the ability of NO-aspirin to inhibit cell growth. Cells were treated with NO-aspirin for 24 h before MTT assay was performed. Data are expressed as percentage of cell growth of control. Results are the mean  $\pm$  S.E.M. of more than three experiments. Statistically significant differences from the corresponding control values are marked by asterisks (\*\*,  $P < 0.01$ ); all other differences are not statistically significant.





**Fig. 13.** Action of NO-aspirin on MAPK signaling. Thickness of arrows indicates the relative effect of NO-aspirin on affected pathways.

had no effect on the ability of NO-aspirin to inhibit the growth of cells in culture. This finding differentiates NO-aspirin from conventional NSAIDs that inhibit colon cancer cell growth. For example, sulindac inhibits ERK1/2 phosphorylation, and overexpression of mitogen-activated protein kinase kinase 1 (activator of ERK) makes HT-29 cells resistant to the known apoptotic effect of sulindac (Shiff et al., 1995; Rice et al., 2004). Sulindac sulfide induces apoptosis in HCA-7 cells and activates ERK and p38; these effects were attenuated by inhibiting ERK or p38 (Sun and Sinicrope, 2005). Similar to ERK1/2, NO-aspirin had little effect on Akt activation, and its inhibition by wortmannin had no effect on the growth of HT-29 cells. Thus, Akt is essentially ruled out as a pathway involved in the mediation of the effect of NO-aspirin.

NO-aspirin treatment of colon cancer cells activated JNK and p38 along with their respective downstream transcription factors, cJun and ATF-2. The activation of these two MAPKs was quantitatively pronounced. NO-aspirin stimulation of p38 seems biphasic, with an initial increase in phosphorylation occurring within the first hour of treatment, and a second much stronger increase at 4 h. These changes are accompanied by changes in the levels of phosphorylated ATF-2, which also display the weakly biphasic pattern of p38 phosphorylation.

The phosphorylation of both isoforms of JNK, JNK1 and JNK2, was increased rapidly in response to NO-aspirin, reaching essentially maximal levels by 30 to 60 min. Of note, the higher NO-aspirin concentration (100  $\mu$ M) led to a more delayed and prolonged induction of phosphorylation of both JNK isoforms. Activation of phosphorylated cJun by the activated JNK was evident at 30 min. There was an interesting dichotomy here with respect to NO-aspirin concentrations. Whereas 10  $\mu$ M NO-aspirin increased cJun phosphorylation progressively for the first 8 h, the 100  $\mu$ M concentration increased the levels of phosphorylated cJun maximally at 30 min. Specific inhibition of p38 or JNK using appropriate inhibitors failed to block the effect of NO-aspirin on cell

growth. In contrast, the dual inhibitor of p38 and JNK did block the effect of NO-aspirin on cell growth, suggesting that both these signaling proteins mediate the effect of NO-aspirin on cell growth.

This conclusion was, however, tentative because it was based on the use of pharmacological inhibitors, which always have the potential of unknown effects beyond the inhibition of their target proteins. The studies using either siRNA for gene silencing or the DNcJun were therefore needed for a thorough assessment of the contribution of p38 and JNK. Whereas transfections with siRNA directed at p38 or cJun alone failed to alter the effect of NO-aspirin on cancer cell growth, the combined silencing of cJun and p38 inhibited significantly the effect on NO-aspirin on cell growth. These findings, being in excellent agreement with the results obtained using pharmacological inhibitors, make essentially certain the conclusion that NO-aspirin needs to stimulate the p38 and JNK pathways to inhibit cell growth. The results with the DNcJun are consistent with this notion as well. The likely huge excess of nonphosphorylatable cJun occupied the AP-1 site blocking signal transduction via either the JNK or p38 pathway.

What remains unknown is the downstream mediators of NO-aspirin on cell growth. The two signaling pathways, p38 and JNK, that are so critical to the cell kinetic effect of NO-aspirin are known to modulate a range of molecules that ultimately determine cell renewal and/or cell death (Bode and Dong, 2004). Such effects include, for example, modulation of cell cycle or of members of the apoptotic pathways. Ongoing work attempts to decipher these interactions. We can, however, speculate that this effect is predominantly through the apoptosis pathway, which seems to be significantly affected by NO-aspirin (Kashfi et al., 2002).

In conclusion, NO-aspirin exerts a profound cell growth inhibitory effect on colon and other cancer cell lines by activating both the p38 and JNK MAPK signaling pathway. These findings have potentially important implications for understanding the mechanism of action of such a promising

chemoprevention agent and also for the design of rational chemoprevention and perhaps chemotherapy approaches.

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**Address correspondence to:** Dr. Basil Rigas, Division of Cancer Prevention, Department of Medicine, Life Sciences Bldg. 06, SUNY at Stony Brook, Stony Brook, NY 11794-5200. E-mail: basil.rigas@sunysb.edu

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