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Nitro-aspirin inhibits MCF-7 breast cancer cell growth: Effects on COX-2 expression and Wnt/β-catenin/TCF-4 signaling

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ABSTRACT

There is current evidence implicating the Wnt/β-catenin/TCF pathway in breast cancer. We investigated the effect of *para*- and *meta*-positional isomers of nitric oxide-releasing aspirin (NO-ASA), and aspirin (ASA) on MCF-7 human breast cancer cell growth and β-catenin/TCF signaling. The *p*- and *m*-NO-ASA isomers strongly inhibited cell growth and β-catenin/TCF transcriptional activity compared to ASA; the IC_{50}s for growth inhibition were 57 ± 4, 193 ± 10 and >5000 μM, and for transcriptional inhibition they were 12 ± 1.8, 75 ± 6.5 and >5000 μM for *p*-, *m*-NO-ASA and ASA, respectively. *p*-NO-ASA reduced the expression of Wnt/β-catenin downstream target gene *cyclin D1*, and total cellular β-catenin levels. COX-2 expression was induced by *p*-NO-ASA, protein kinase C inhibitors reversed this induction. *p*-NO-ASA blocked the cell cycle transition at S to G_2/M phase. These studies suggest a targeted chemopreventive/chemotherapeutic potential for NO-ASA against breast cancer.

1. Introduction

The second leading cause of cancer deaths amongst women in the US is breast cancer [1]. Chemoprevention strategies with selective estrogen receptor modulators such as tamoxifen and raloxifene have a well-established role in preventing breast cancer [2,3]. However, these agents have limitations due to their efficacy primarily for estrogen receptor positive [ER(+)] breast cancer and development of tamoxifen resistance [4,5]. The concern in using endocrine therapy for chemoprevention and/or chemotherapeutics has encouraged investigation of alternative chemopreventive compounds that would restore normal growth control to a cancerous cell population by modifying aberrant signaling pathways, inducing apoptosis, inhibiting proliferation, or a combination of these.

The Wnt/β-catenin signaling pathway is highly regulated and has important functions in development, tissue homeostasis, and regeneration. Not only mouse model systems clearly demonstrate that activated Wnt/β-catenin signaling leads to mammary tumorigenesis [6,7] but that aberrant activation of this signaling pathway can lead to the development of many human cancer types [8,9] including the breast [10]. Therefore, the Wnt/β-catenin signaling pathway constitutes a *bona fide* chemotherapeutic target.

Considerable body of evidence strongly suggests that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of and mortality from colon cancer by about half and constitute the prototypical colon cancer chemopreventive agents [11]. The use of NSAIDs may also be associated with reduced risk for breast cancer, but results from these studies of the association have been inconsistent. Several studies have reported a reduced risk [12,13] while others have failed to find any association or have even suggested an increased risk [14,15]. However, a recent exhaustive meta-analysis provides evidence that NSAID use is associated with reduced risk for breast cancer [16]. Regular NSAID use however, may be associated with serious side effects including gastrointestinal and renal [17]. The clinical usefulness of NSAIDs together with their serious side effects prompted the search for a "better NSAID". This led to the development of selective cyclooxygenase-2 (COX-2) inhibitors (Coxibs) and nitric oxide-releasing NSAIDs (NO-NSAIDs). Coxibs are as effective as traditional NSAIDs for the indications for which they have been approved, however, the recent withdrawal of rofecoxib and valdecoxib, two COX-2 specific inhibitors, on account of their significant cardiovascular toxicity has raised concerns about this class of compounds [18]. NO-NSAIDs are emerging as a novel class of chemoprevention agents

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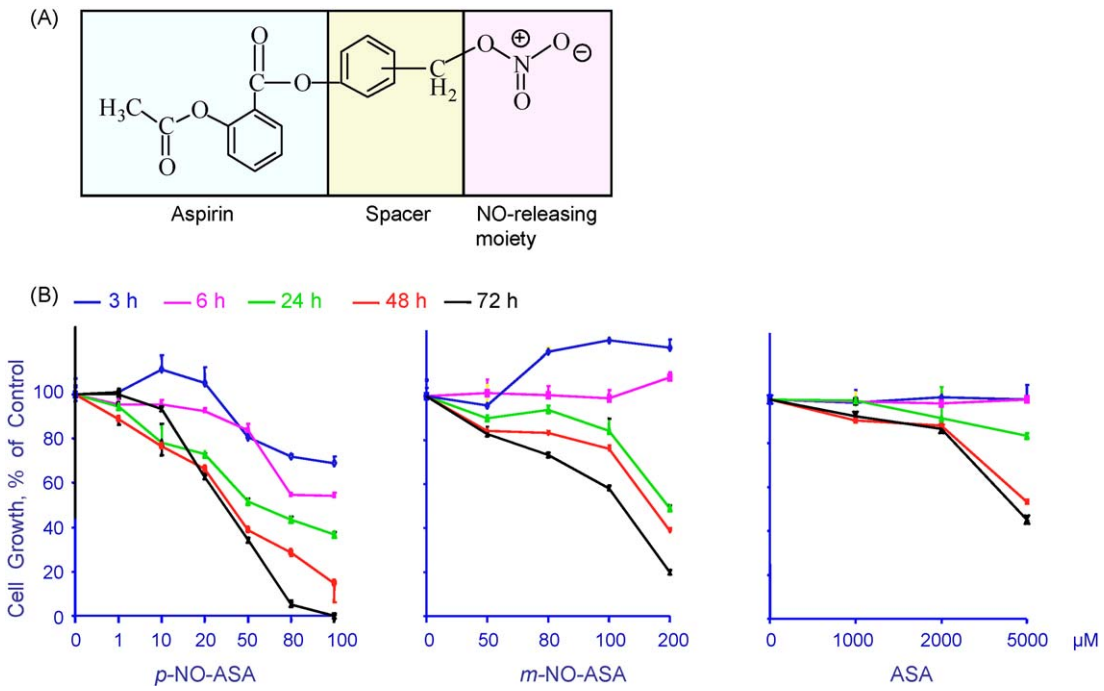

Fig. 1. Concentration- and time-dependent inhibition of MCF-7 cell growth by ASA and the positional isomers of NO-ASA. Cells were treated with various concentrations of *p*- or *m*-NO-ASA or ASA as described in Section 2. Cell numbers were determined at 24, 48, and 72 h. Results represent means ± SEM of three different experiments performed in triplicate. See Table 1 for IC_{50} values ± SEM.

[19–21]. They consist of a traditional NSAID and a NO-releasing moiety linked to it via a chemical spacer. All NO-NSAIDs studied to date appear free of appreciable adverse side effects, while they retain beneficial activities of the parent compounds. Amongst the various NO-NSAIDs, NO-aspirin (NO-ASA, Fig. 1) appears to be the most effective/potent in inhibiting the growth of various cancer cell lines [19]. NO-ASA has also shown to have strong chemopreventive properties in various animal models of cancer [22–24]. In our analysis of its molecular targets using colon cancer cell lines, we have demonstrated TCF/β-catenin signaling and its elements to be highly affected [25–27].

In recent years, overexpression of COX-2 and its subsequent COX-2-derived prostaglandin E_2 (PGE_2) has been implicated in the development of various types of cancer [28]. Not surprising, COX-2 inhibition has been a major target for prevention of cancer. Alternative interpretation of the data has suggested that COX-2 inhibitors may work through COX-independent targets [29]. The underlining mechanism(s) for the induction of COX-2 has not been totally elucidated. Recent data suggest that the induction of COX-2 by a variety of agents may involve a calcium/protein kinase C (PKC)-dependent pathway [30–32]. We recently reported that even though NO-ASA inhibited HT-29 human colon cancer cell growth, it induced COX-2 expression and that this induction was accompanied by increased PGE_2 production [27].

Here we report our observation on the effects of NO-ASA in MCF-7 breast cancer cell line with respect to COX-2 expression, cell growth inhibition, effects on the Wnt/β-catenin/TCF signaling pathway, including expression of its downstream target gene, *cyclin D1*.

2. Materials and methods

2.1. Reagents and cell culture

NO-ASA, *para* isomer, [2-(acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester]; and the *meta* isomer, [2-(acetyloxy)benzoic acid 3-(nitrooxy methyl)phenyl ester] were synthesized and purified by us according to previously published methods [33]. ASA was obtained from Sigma (St. Louis, MO). Stock solutions (100 mM) were made in DMSO; final DMSO concentration was adjusted in all media to 1%. MCF-7 cells from ATCC (HTB-22 Manassas, VA) were grown according to supplier's instructions.

2.2. Plasmids, transient transfection and reporter gene assay

Transient transfection was performed using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Briefly, 10^5 cells were transfected with 800 ng of luciferase reporter constructs TOPflash or FOPflash (Upstate Biotechnology, Lake Placid, NY) and 200 ng of pSV-β-gal vector as internal control. TOPflash contains three copies of the TCF/LEF binding site (AAGATCAAAGGGGGT) upstream of a TK minimal promoter. FOPflash contains a mutated TCF/LEF binding site (AAGGC-CAAAGGGGGT). Two hours post-transfection, cells were treated for 18 h with NO-ASA or ASA. Luciferase activity was measured per manufacturer's instructions and β-galactosidase activity was measured using standard protocols. The activity was normalized to β-gal values and expressed as relative fold compared to control.

2.3. Cell growth inhibition assay

The growth inhibitory effect of NO-ASA on MCF-7 cells was measured using a colorimetric MTT assay kit (Roche, Indianapolis, IN). Briefly, cells were plated in 96-well plates at a density of 10×10^3 cells/well and, following overnight incubation, NO-ASA was added to the culture medium. At indicated time periods of treatment, viable cells were quantified with MTT substrate according to the manufacturer's instructions. Relative cell growth was shown compared with the corresponding control cells. Growth inhibition was expressed as percentage of the corresponding control.

2.4. Western blot analysis

After treatment with NO-ASA or vehicle, cells were harvested and lysed in buffer containing 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA and 0.5% IGEPAL, 10% glycerol in the presence of protease inhibitors. Proteins were fractionated by SDS-PAGE and transferred to supported nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Primary mouse monoclonal antibody anti-β-catenin, 1:1000 (BD-Transduction Laboratories, San Jose, CA), anti-COX-2, 1:1000 (Caymen Chemicals, Ann Arbor, MI); rabbit polyclonal anti-cyclin D1, 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Secondary antibodies conjugated to horseradish peroxidase (1:4000) were from Sigma. Immunoreactive protein was detected using chemiluminescence substrates (Pierce Chemicals, Rockford, IL). In some experiments, after seeding the cells overnight, they were then serum starved for 24 h followed by treatment with NO-ASA (20 μM) or the vehicle. In these experiments, the cells were pretreated for 1 h with either of the two different PKC inhibitors, Ro-318220 (1 μM) [34] or GF 109203X (10 μM) [35] (Sigma–Aldrich, St. Louis, MO) before addition of NO-ASA or vehicle. After treatment, cells were harvested and COX-2 expression determined by immunoblotting.

2.5. Cell proliferation and cell cycle analysis

Cell cycle phase distributions of control and treated MCF-7 cells were obtained 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 listmode files. Data were analyzed on a Coulter XL Elite Work station using the Software programs Multigraph™ and Multicycle™.

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. Cell were washed again 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. The percentage of cells in G_0/G_1, G_2/M, and S phases was determined form DNA content histograms. The induction of apoptosis was determined by the presence of a sub-diploid (sub-G_0/G_1) peak in DNA content histograms. To assess proliferation, cells were washed twice in PBS/1% FBS, pelleted, and resuspended in 50 μL of a 1:10 dilution of the anti-PCNA primary antibody (PC-10; all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/1% FBS for 60 min at room temperature. Nonspecific IgG1/IgG2 was used as an isotypic control.

2.6. Statistics

Data are presented as means ± SEM for at least three different sets of plates and treatment groups. Statistical comparison amongst the groups was performed using Student's *t*-test.

3. Results

3.1. NO-ASA strongly inhibits the growth of MCF-7 cells

We examined whether NO-ASA affects the growth of MCF-7 breast cancer cells in culture. Cells were treated with several concentrations of NO-ASA isomers; *para* and *meta* (*p*-NO-ASA and *m*-NO-ASA), respectively, or with aspirin (ASA) and compared to an untreated control. Time and concentration effects on MCF-7 cell growth were studied with time intervals up to 72 h. Fig. 1 shows both NO-ASA isomers strongly inhibit the growth of MCF-7 cells as compared to ASA in a concentration and time dependent manner.

Table 1
IC_{50} (μM) values for positional isomers of NO-ASA in MCF-7 cells. Cells were treated with various concentrations of the test agents for 24–72 h, from which cell numbers and IC_{50} values were calculated. Results are mean ± SEM of three different experiments done in triplicate.

Compound	IC_{50} (μM)		
	24 h	48 h	72 h
Aspirin	>5000	>5000	4650 ± 230
p-NO-ASA	57 ± 4*	38 ±2*	33 ± 2*
Ratio	*>88*	>132	*141 ± 3*
m-NO-ASA	193 ± 10*	170 ± 7*	122 ± 15*
Ratio	*>26*	*>29*	*38 ± 1*

* $P < 0.01$ compared to ASA.

The IC_{50}s for growth inhibition were reduced in a time-dependant manner (Table 1). At 24, 48, and 72 h, the IC_{50}s of *p*-NO-ASA were 57 ± 4, 38 ± 2, and 33 ± 2 μM, respectively. For *m*-NO-ASA, the corresponding IC_{50}s were, 193 ± 10, 170 ± 7 and 122 ± 15 μM. In contrast, the IC_{50}s for ASA, were higher than 5000 μM at 24 and 48 h and 4650 ± 230 μM at 72 h. The ratio of IC_{50} values (traditional ASA/NO-ASA) at 24 h indicated that the *p*-NO-ASA to be >88-fold and *m*-NO-ASA to be >26-fold more potent than ASA, and at 48 h, the *p*-NO-ASA to be >132-fold and *m*-NO-ASA to be >29-fold more potent than ASA. The only enhancement that could be calculated accurately was for the 72 h time point, 141-fold for *p*-NO-ASA and 38-fold for *m*-NO-ASA. An intriguing observation was a transient increase in growth (approximately 10–20%) at 3 h of treatment with *p*-NO-ASA (10 and 20 μM) and *m*-NO-ASA (80–200 μM); the implication of this is not clear (Fig. 1).

3.2. NO-ASA strongly inhibits β-catenin/TCF transcriptional activity

Beta-catenin/TCF transcriptional activity is known to regulate proliferative activities of breast epithelial cells notably by regulating the expression of the downstream target gene *cyclin D1* [36]. We determined the effect on β-catenin/TCF transcriptional activity by *p*- and *m*-NO-ASA compared to ASA. MCF-7 cells were transiently transfected as described, followed by treatment with of *p*- and *m*-NO-ASA isomers (1–200 μM) or ASA (1000–5000 μM) for 24 h, and luciferase reporter activity was measured. The *p*- and *m*-isomers showed strong inhibitory activity as compared to ASA (Fig. 2). At 10 and 20 μM concentration the *p*-isomer reduced the transcriptional activity by 45 ± 2 and 63 ± 1%, respectively, compared to vehicle-treated control. The *m*-isomer was less potent, similar to our previous demonstration using colorectal cancer cell lines [27]. It is important to note that *p*-NO-ASA was also effective at the very low concentration of 1 μM, reducing the transcriptional activity by about 33% compared to control. The IC_{50}s determined for inhibition of β-catenin/TCF transcriptional activity were 12 ± 1.8 μM for *p*-NO-ASA, 75 ± 6.5 μM for *m*-NO-ASA, and >5000 μM for ASA.

3.3. NO-ASA reduces β-catenin protein levels

Since β-catenin/TCF-4 transcriptional activity was reduced by NO-ASA isomers, the possibility of downregulation of β-catenin expression by NO-ASA was examined by assessing its cellular levels following *p*-NO-ASA treatment. Lysates of MCF-7 cells were made after treatment with *p*-NO-ASA for 24 h at different concentrations that exhibited β-catenin/TCF inhibition in the reporter assay. As shown in Fig. 3A and B, *p*-NO-ASA reduced the levels of β-catenin at 20 and 40 μM by 28 ± 3% ($P < 0.05$) and 62 ± 4% ($P < 0.01$), respectively. ASA at 1 mM and *m*-NO-ASA up to 400 μM had no effect on β-catenin expression. Therefore, we used *p*-NO-ASA for further studies.

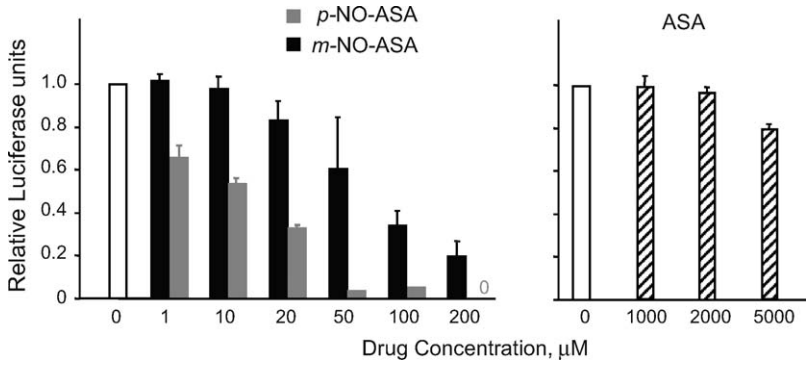

Fig. 2. Concentration dependent inhibition TCF-4 responsive reporter gene by ASA and NO-ASA isomers. MCF-7 cells, cotransfected with luciferase reporter plasmids (TOP or FOP) and the pSV-β-gal as in Section 2 were treated with various concentrations of ASA and NO-ASA for 24 h. Relative TCF activity (fold) of treated cells is shown (DMSO control was set as 1); values are mean ± range of two different experiments performed in triplicate. (*Left*) *p*- and *m*-NO-ASA strongly inhibit TCF signaling more than ASA (*right*).

3.4. NO-ASA reduces cyclin D1 levels

Of the several of genes whose transcription is regulated by β-catenin/TCF-4, *cyclin D1* plays an important role in the process of carcinogenesis [36]. Therefore, we investigated whether *p*-NO-ASA affected the Cyclin D1 levels in MCF-7 cells (Fig. 3C and D). After 24 h of treatment, Cyclin D1 protein expression was down-regulated. The downregulation occurred between 10 and 20 μM *p*-NO-ASA and remained at that level for higher concentrations of *p*-NO-ASA. It is noteworthy that this downregulation occurred close to the IC_{50} for inhibition of β-catenin/TCF-4 transcriptional activity.

3.5. Cell cycle and cell proliferation effects

To elucidate the mechanism underlying the effect of NO-ASA on cell growth we determined their effect on cell kinetic parameters as follows. The effects of *p*-NO-ASA on cell cycle were assessed to determine whether the growth inhibition was due to alterations in the different phases. MCF-7 cells were treated with *p*-NO-ASA for

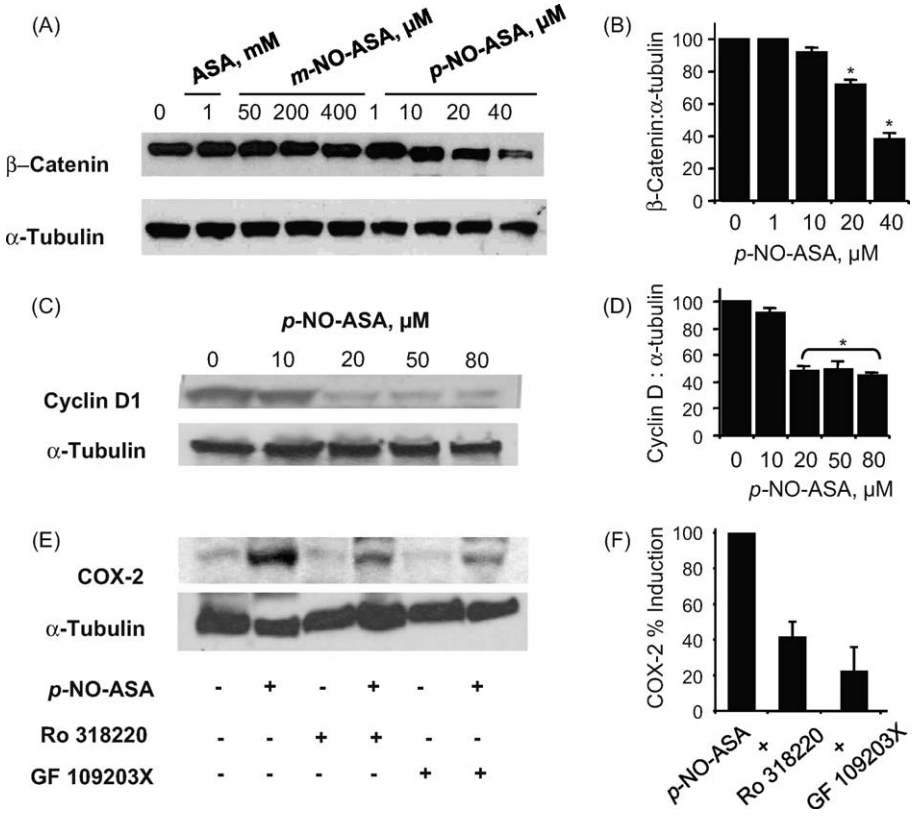

Fig. 3. The *para* isomer of NO-ASA reduces β-catenin and cyclin D1 levels and induces COX-2 expression. MCF-7 cells treated with increasing concentrations of ASA, *m*- and *p*-NO-ASA for 24 h were analyzed for total β-catenin expression by immunoblot of lysates (panel A). Densitometry evaluation of levels of β-catenin and α-tubulin performed from three such immunoblots is shown in panel B. ASA and *m*-NO-ASA had no effect on β-catenin expression, however, *p*-NO-ASA reduced β-catenin levels dose dependently. The protein levels of cyclin D1 were also reduced by *p*-NO-ASA (panel C) and as shown for three immunoblot evaluations is panel D. $^*P < 0.05$ compared to untreated controls. For induction of COX-2 studies, MCF-7 cells were serum deprived for 24 h and then treated with 20 μM *p*-NO-ASA for 24 h. In some experiments the cells were preincubated for 1 h with either of the PKC inhibitors Ro 318220 or GF 109203X before NO-ASA treatment. The cells were then harvested and COX-2 expression determined by immunoblotting as described in Section 2 (panel E, representative of two independent experiments). Panel F, COX-2 levels are normalized to α-tubulin. Results are the mean ± range of two independent experiments.

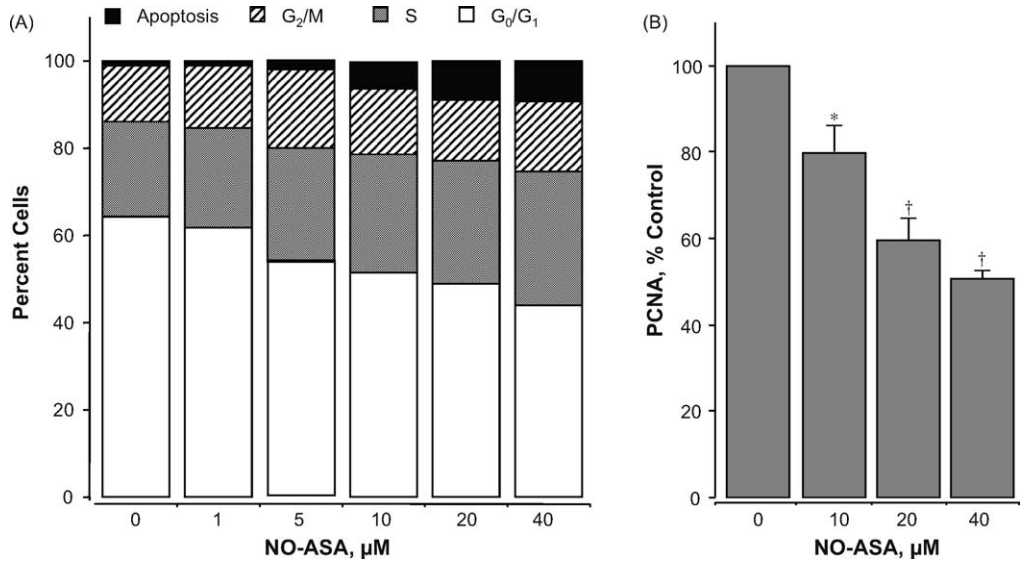

Fig. 4. Effect of *p*-NO-ASA on cell proliferation and cell cycle in MCF-7 cells. Cells were treated for 24 h with various concentrations of *p*-NO-ASA, and their cell cycle phase distribution was determined by flow cytometry, as described under Section 2 (panel A). Results are representative of two different experiments. This study was repeated twice generating results within 10% of those presented here. Pane B, cells were treated with *p*-NO-ASA at the concentrations indicated for 24 h after which PCNA expression was determined by flow cytometry and expressed as percentage positive cells as described in Section 2. Results are mean ± SEM of three different experiments. $^{*}P < 0.05$; $^{\dagger}P < 0.01$ compared with untreated cells.

24 h at different concentrations. NO-ASA induced a concentration-dependent block of the S to G_2/M transition (Fig. 4). Following 24 h of treatment with 5 μM *p*-NO-ASA, G_0/G_1 changed from 64 to 54% and S from 21 to 26%, whereas G_2/M did not change appreciably. These changes became more pronounced at 40 μM (G_0/G_1 from 64 to 44% and S from 21 to 30%, whereas G_2/M showed a minor increase from 13 to 16%). Concomitantly, the population of apoptotic cell increased from 1 to 10% in a dose-dependent manner. At 24 h, *p*-NO-ASA reduced PCNA expression in a dose-dependant manner. It is noteworthy that the PCNA reduction was 58 ± 3% at 40 μM *p*-NO-ASA which is close to its IC_{50} for cell growth inhibition.

3.6. NO-ASA induces COX-2 expression

Our previous data had shown that treatment of HT-29 human colon and BxPc-3 human pancreatic cancer cells with *p*-NO-ASA (20 μM) induced COX-2 expression [27] but we offered no explanation for these observations. Here, we also found that *p*-NO-ASA (20 μM) induces COX-2 expression in MCF-7 breast cancer cells. Since recent data suggest that the induction of COX-2 may involve a calcium/PKC-dependent pathway [30–32], here we used two different PKC inhibitors, Ro 318220 and GF 109203X, both of which are inhibitors of α, β, and γ isoforms of PKC [37], to evaluate whether the observed induction of COX-2 expression by NO-ASA was PKC dependent. MCF-7 cells were seeded at a density of 3×10^5 cells/cm^2 and grown overnight. Cells were then serum deprived for 24 h followed by treatment with *p*-NO-ASA (20 μM) or the vehicle. In some experiments, the cells were pretreated for 1 h with either of two different PKC inhibitors before addition of *p*-NO-ASA or vehicle. After treatment, cells were harvested and COX-2 expression determined by immunoblotting. Fig. 3E and F shows that *p*-NO-ASA at a concentration of 20 μM which is below its IC_{50} for cell growth inhibition, induces COX-2 expression. Preincubation of the MCF-7 cells with Ro 318220 or GF 109203X partially reversed the *p*-NO-ASA-mediated induction of COX-2 by approximately 60 and 80%, respectively. This suggests that *p*-NO-ASA exerts, in part, a PKC-dependent induction of COX-2 expression. *p*-NO-ASA had no effect on COX-1 expression (data not shown). In order to determine the effects of PKC inhibition on the growth inhibitory properties of *p*-NO-ASA, we either preincubated MCF-7 cells for 1 h with 10 μM GF 109203X and then challenged the cells with different concentrations of *p*-NO-ASA for 24 h; or co-treated the cells with 10 μM GF 109203X and different concentrations of *p*-NO-ASA for 24 h. In both cases, the PKC inhibitor had little to no effect on the growth inhibitory properties of *p*-NO-ASA (data not shown).

4. Discussion

These studies highlight several significant points with respect to the mechanism of action of NO-ASA. Our previous studies had shown that NO-ASA inhibited the growth of various cultured human cancer cells, suggesting a tissue type-independent effect [19]. In this study we have extended these observations to include MCF-7 breast cancer cells. In agreement with our earlier reports, this study shows that NO-ASA was also more potent than ASA in inhibiting MCF-7 cell growth, therefore, giving more credence to our earlier findings. The ratio of IC_{50} values (ASA/NO-ASA) ranged from >88- to 141-fold for *p*-NO-ASA and from, >26- to 38-fold for *m*-NO-ASA over a time period of 24–72 h (Table 1). Similar enhanced potency of NO-ASA was exhibited in a variety of human cancer cell lines such as colon, pancreas, prostate as well as Jurkat T leukemia cells [19,26].

NO-ASA has a profound effect on the Wnt/β-catenin signaling pathway. In MCF-7 breast cancer cells, the inhibition of this pathway took place at concentrations that were significantly below its IC_{50} for growth inhibition. For example, *p*-NO-ASA strongly inhibited β-catenin/TCF-4 transcriptional activity and cell growth with IC_{50}s of 12 ± 1.8, and 57 ± 4 μM at 24 h, respectively. If such an effect occurs in intact organisms such as experimental models of cancer or in humans, it may explain much of the chemopreventive effect of NO-ASA demonstrated in animal models of cancer studied to date [22–24]. Since the inhibition of the transcriptional event occurred at a much lower concentration than its IC_{50} for growth inhibition, this strongly suggests that molecular events occur early leading to cell growth inhibition.

Including the present study, *p*-NO-ASA has been shown to modulate the β-catenin signaling pathway in three different cancer cell lines of different tissue origins [25,26,38]. In Jurkat T

cells, *p*-NO-ASA degraded total β-catenin levels in a dose- and time-dependent manner [26]. Similarly, in SW480 colorectal cancer cells, *p*-NO-ASA (50–80 μM) degraded β-catenin, whereas lower concentrations (1–20 μM) blocked the formation of β-catenin/TCF complexes; thereby inhibiting β-catenin/TCF transcriptional activity by a dual mechanism [25,38]. In MCF-7 cells, *p*-NO-ASA moderately reduced total levels of β-catenin levels in a concentration-dependant manner. Taken collectively, we conclude that *p*-NO-ASA-mediated inhibition of β-catenin/TCF signaling and that the effects on β-catenin are not cell-type-restricted. Regarding the intricacies of β-catenin degradation, *p*-NO-ASA was shown to induce the expression of activated caspase-3 and was associated with substantial degradation of β-catenin in Jurkat and SW480 cells [25,38]. However, MCF-7 cells are caspase-3-depleted owing to a 47-base pair deletion within exon 3 of the *CASP-3* gene, and therefore, lack caspase-3, -6, and -7 cleavage activities [39]. This partly explains the moderate reduction of β-catenin in these cells and suggests other caspases or mechanisms may be involved.

One of the significant downstream genes that are dependent on β-catenin/TCF-4 signaling is *cyclin D1*, which has been implicated in carcinogenesis [8]. The inhibition of this signaling pathway by low concentrations of *p*-NO-ASA, which was associated with reduced cyclin D1 expression, suggests that it may represent an important disruption in the carcinogenic process. The sharp drop in cyclin D1 level occurred at 10–20 μM *p*-NO-ASA although a modest decrease in β-catenin levels occurred only at 40 μM. It may be envisaged that the decrease in cyclin D1 levels at lower concentrations may be possible due to disruption of the β-catenin/TCF-4 complexes at low concentrations of *p*-NO-ASA, which was demonstrated in studies in other cell lines [25,38].

NO-ASA inhibits the growth of cultured MCF-7 breast cancer cells through a complex effect on cell kinetics. This effect involves cell renewal, cell death and the transitions of the cell through the cell cycle phases. It is noteworthy that the contribution of each of these parameters is different in cells of different tissue origin. For example, our previous work with HT-29 human colon and MIA PaCa2 human pancreatic cancer cells indicated that the induction of cell death appeared to be a more prominent effect of NO-ASA rather than inhibition of proliferation [19,40]. The transition of cells through the cell cycle was blocked from G_2M to G_0/G_1 in the pancreatic cells, and a G_0–G_1 to S block was observed for the colon cancer cells. Therefore, based on the different increases in different cell cycle phases in MCF-7 cells, the possibility of multiple blockades cannot be ruled out. Also, in the present study, NO-ASA caused only a very modest induction of apoptosis in the MCF-7 breast cancer cells, while it significantly inhibited cell proliferation. This observation is inline with a recent report describing the effects of sulindac in this cell line [41]. Absence of an intact *CASP-3* gene in MCF-7 cells may lead to the modest apoptosis and therefore, stronger effects on cell cycle phases. In our present study, the transition of cells through the cell cycle was blocked from S to G_2/M, highlighting a different block to that we reported for colon and pancreatic cells. The fraction of cells present in the S phase went up consistently from 21 to 30% in a concentration-dependant manner, however, the cells in G_2/M phase increased only from 13 to 18% at 5 μM *p*-NO-ASA and then decreased to 16% at 40 μM. Thus, replicating cells in the S phase did not enter into the G_2 and M phase proportionately, reflecting a block between the G_2 and M phase, i.e., the G_2/M phase. Traditional NSAIDs are known to affect cell cycle transition through changes in proteins that control them [42]. It has also been shown that endogenous NO can modulate the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ in pancreatic cell lines [43]. Thus NO-ASA, bearing ASA and a NO-releasing moiety may affect the regulation of the cell cycle through its components. In addition, it is possible that such a complex process of growth inhibition by *p*-NO-ASA may include cell cycle effects, mediated in part, through COX-2 induction or PKC activity, and therefore, merit further investigation.

Regarding the effect of NO-ASA on COX that transform arachidonic and other fatty acids into eicosanoids, only the expression of COX-2 was induced by 24 h, while that of COX-1 was not affected. This is in agreement with our previous report using colon and pancreatic cancer cell lines [27]. However, it should be noted that the induction of COX-2 by NO-ASA is inconsistent with the accepted model of its regulation by the Wnt pathway. Also, induction of COX-2 in general is considered to contribute to carcinogenesis. Our finding can be interpreted either as indicating that this is a change restricted to cell culture systems or that COX-2 may play a far more complex role in carcinogenesis than currently understood. Our present data also strongly suggest that the induction of COX-2 by NO-ASA is at least in part PKC dependent. However, the observed growth inhibition by NO-ASA appears to be independent of PKC activity.

In conclusion, our data demonstrate that NO-ASA strongly inhibited cell proliferation in MCF-7 human breast cancer cells, at least in part, by reducing the expression of β-catenin and inhibition of β-catenin/TCF dependent transcriptional activity, leading to reduced expression of its target gene *cyclin D1*. This provides a rational targeted approach for prevention/treatment.

Acknowledgments

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