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Nitric Oxide-Donating Nonsteroidal Anti-Inflammatory Drugs Inhibit the Growth of Various Cultured Human Cancer Cells: Evidence of a Tissue Type-Independent Effect

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ABSTRACT

The novel nitric oxide (NO)-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs), which are safer than their NSAID counterparts, inhibit the growth of colon cancer cells with far greater potency than traditional NSAIDs. We examined whether NO-NSAIDs inhibit the growth of cancer cells arising from other human tissues. Human pancreatic, colon, prostate, lung, and tongue cancer cell lines were treated with NO-aspirin, -sulindac, -ibuprofen, and -indomethacin or their traditional counterparts. We determined IC_{50} values, cell proliferation, apoptosis, cell cycle, cyclooxygenase (COX) protein levels, and morphological changes (light and electron microscopy). All NO-NSAIDs inhibited the growth of all cancer cell lines studied. The potency of NO-NSAIDs was 11- to 6000-fold greater than that of their counterparts (except for the effect of sulindac on lung cancer cells). NO-aspirin was consistently the most potent NO-NSAID in all cell lines tested (except for the lung cancer cell line), sometimes in excess of 100-fold over the other three NO-NSAIDs. NO-NSAIDs inhibited cell proliferation, induced apoptosis, and altered cell cycle phase distribution (G_2/M to G_0/G_1 block). All altered cellular morphology, whereas NO-aspirin induced nuclear disintegration ("atypical" cells) established by electron microscopy. NO-aspirin showed similar effects on two pancreatic cancer cell lines, BxPC-3 (expresses COX) and MIA PaCa-2 (no COX expression), suggesting a COX-independent effect. NO-NSAIDs showed a tissue-type-independent effect. Their pleiotropic effects involve cell renewal, cell death, and cell cycle phase transitions. These results raise the possibility that NO-NSAIDs possess chemopreventive and/or chemotherapeutic activity against a wide variety of human cancers.

NO-donating NSAIDs (NO-NSAIDs) represent a promising development in the prevention and/or treatment of cancer. They consist of a traditional NSAID to which a group donating NO has been covalently attached via an aromatic or aliphatic spacer (Fig. 1). Emerging data indicate that these compounds combine the chemopreventive properties of traditional NSAIDs against cancer with enhanced safety and efficacy (Fiorrucci et al., 2002; Rigas and Williams, 2002). In the case of NO-donating aspirin (NO-ASA), for example, we have reported it to be between 2540- to > 5000-fold more potent than traditional ASA in suppressing colon cancer cell growth (Williams et al., 2001). Such enhancement has been attributed primarily to the presence of the NO donating moiety on the new modified NSAID molecule and to a lesser degree to the spacer molecule (Kaza et al., 2002). Studies with NO-ASA using an animal model of colon cancer demonstrated that it is more efficacious than ASA in preventing the formation of aberrant crypt foci, a precursor of colon cancer (Bak et al., 1998).

Our initial work was focused on colon cancer cell lines; the colon is the best-known target organ for demonstrating the chemopreventive effect of traditional NSAIDs. We examined the effect of three NO-NSAIDs, namely NO-ASA, NO-ibuprofen, and NO-sulindac on cell kinetics (Williams et al., 2001). All three were more potent than their traditional parent NSAIDs in inhibiting the growth of cultured colon cancer

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ABBREVIATIONS: NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; ASA, aspirin; NCX1102, (*Z*)-5-fluoro-2-methyl-1-{[4-(methylsulfinyl)phenyl]methylene}-1*H*-indene-3-acetic acid 4-(nitrooxy)butyl ester; NCX2210, *trans*-3-{4-[α-methyl-4-(2-methylpropyl)benzeneacetyleproxy]-3-methoxyphenyl}-2-propenoic acid 4-nitrooxy)butyl ester; NCX 2121, (*S*)-*N*-acetyl-[1-(4-(chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-acetyl]-cysteine 4-(nitroxybutyl) ester; NCX4040, 2-(acetyloxy)benzoic acid 4-(nitrooxy-methyl)phenyl ester; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PCNA, proliferating cell nuclear antigen; DAPI, 4,6-diamidino-2-phenylindole; COX, cyclooxygenase.

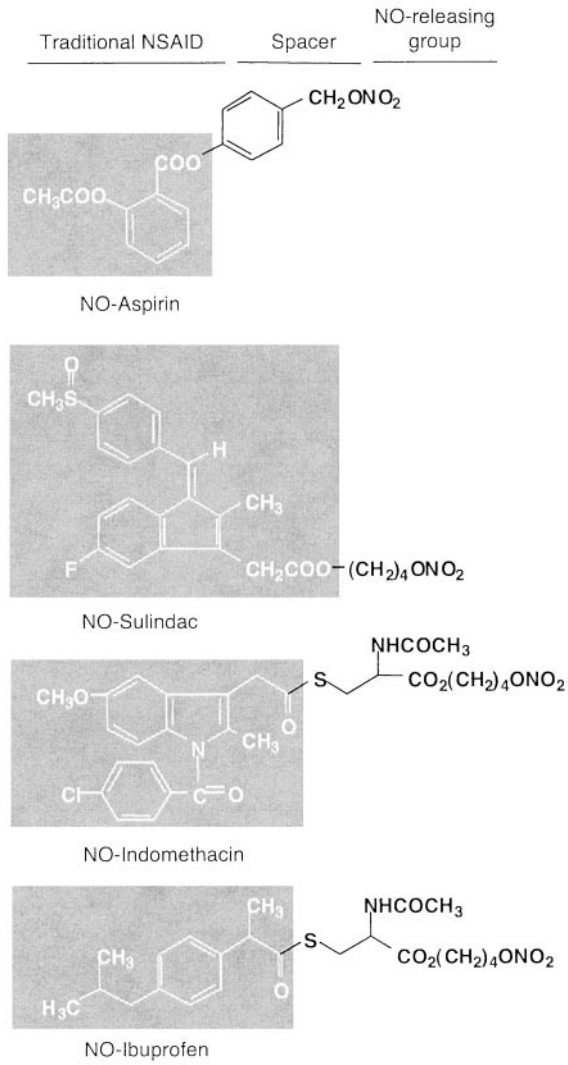

Fig. 1. The chemical structures of NO-NSAIDs. The three structural components of NO-aspirin (salicylic acid derivative), NO-sulindac and NO-indomethacin (indole and indene acetic acids), and NO-ibuprofen (arylpropionic acid derivative) are indicated; the traditional NSAID is shown in the shaded box; the spacer molecule links the traditional NSAID to $-NO_2$, which can release NO.

cells. They achieved this through a combined effect on cell proliferation (inhibition), on cell death (increase), and on cell cycle phase transitions (G_1 to S block). These data raised two questions of specificity that are even more significant given the pharmacological attributes of this novel class of compounds. They concern 1) the "target tissue specificity", i.e., whether NO-NSAIDs display these novel properties in cells other than colon cancer cells, and 2) the "compound specificity", i.e., which of the various members of the large NSAID family display these novel properties. This article addresses both issues, although its focus is mainly on the former.

The question of tissue specificity is very important not only from a mechanistic point of view but also because of its significant practical implications. If NO-NSAIDs manifest in other tissues the powerful effect they showed on colon cancer cells, then tumors arising from these tissues may be appropriate chemopreventive and/or therapeutic targets of these compounds. To this end, we have assessed in detail the effect of four NO-NSAIDs representing three structural classes (salicylic acid derivatives, indole and indene acetic acids, and arylpropionic acid derivatives) on various human cancer cell lines. These cell lines are both of adenomatous (colon, pancreatic, lung, and prostate) and squamous (tongue) cell origin. Our results establish the remarkable potency of these compounds on a wide variety of tissue targets. In addition, they demonstrate the versatility and suggest the strong clinical potential of these compounds, and they also indicate that they modulate a mechanism(s) common to various cancers, which is apparently critical to their growth.

Materials and Methods

Cell lines. MIA PaCa-2 and BxPC-3 human pancreatic cancer, LNCAP human prostate cancer, A549 human lung cancer, HT-29 and HCT-15 human colon adenocarcinoma, and SCC-25 human tongue squamous cell carcinoma cell lines were obtained from American Type Tissue Collection (Manassas, VA). All cells lines were grown as monolayers. The pancreatic cells were grown in Dulbecco's modified Eagle's medium, the prostate and the HCT-15 colon cells were grown in RPMI 1640 medium, the lung cells were grown in F-12, the colon HT-29 cells were grown in McCoy 5A medium, and the SCC-25 in a 1:1 mixture of F-12K and Dulbecco's modified Eagle's medium. All media were supplemented with 10% fetal calf serum (Mediatech, Herndon, VA), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Invitrogen, Carlsbad, CA). Cells were seeded on culture dishes at a density of 25×10^3 cells/cm^2 and incubated at 37°C in 5% CO_2 and 90% relative humidity. Single cell suspensions were obtained by trypsinization (0.05% trypsin/EDTA), and cells were counted using a hemacytometer. Viability was determined by the trypan blue dye exclusion method.

Reagents. NO-sulindac [NCX1102; (*Z*)-5-fluoro-2-methyl-1-{[4-(methylsulfinyl)phenyl]methylene}-1*H*-indene-3-acetic acid 4-(nitrooxy)butyl ester], NO-ibuprofen [NCX2210; *trans*-3-{4-[α-methyl-4-(2-methylpropyl)benzeneacetylepoxy]-3-methoxyphenyl}-2-propenoic acid 4-nitrooxy)butyl ester], NO-indomethacin [NCX2121; (*S*)-*N*-acetyl-[1-(4-(chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-acetyl]-cysteine 4-(nitroxybutyl) ester], and NO-aspirin [NCX4040; 2-(acetyloxy)benzoic acid 4-(nitrooxy-methyl)phenyl ester] were a gift from Nicox, SA (Sophia Antipolis, France). Stock (100 mM) solutions of NO-NSAIDs and NSAIDs were prepared in dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ). Traditional NSAIDs were obtained from Sigma-Aldrich (St. Louis MO). All compounds were added to the culture medium immediately before plating. The final dimethyl sulfoxide concentration was adjusted in all media to 1%.

Flow Cytometry. Cell cycle phase distributions of control and treated colon cancer cell lines were obtained using a Coulter Profile XL equipped with a single argon ion laser (Beckman Coulter, Inc., Fullerton, CA). For each subset, we analyzed >10,000 events. All parameters were collected in list-mode files. Data were analyzed on an XL Elite Workstation (Beckman Coulter, Inc.) using the Software programs Multigraph and Multicycle.

Cell Proliferation and Cell Cycle Analysis. Cells (0.5×10^6) were fixed in 100% methanol for 10 min at −20°C, pelleted (5000 rpm × 10 min at 4°C), resuspended and incubated in PBS containing 1% FBS/0.5% Nonidet P-40 on ice for 5 min. Cells were washed twice in PBS/1% FBS, pelleted and resuspended in 50 μl of a 1:10 dilution of the anti-PCNA primary antibody (PC-10; all antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/1% FBS for 60 min at room temperature. Nonspecific IgG_1/IgG_2 was used as an isotypic control. Cells were then washed and incubated with goat-anti-mouse-phycoerythrin antibody (diluted 1:50) for 60 min at room temperature in the dark. Cells were washed again in 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.

Assays for Apoptosis. The induction of apoptosis was determined by the presence of a subdiploid (sub-G_0/G_1) peak in DNA content histograms obtained by flow cytometry, as described above, and by fluorescence microscopy of cells stained with 4,6-diamidino-2-phenylindole (DAPI; Accurate Chemical, Westbury, NY). For each sample, at least five fields were examined. The morphological criteria used to identify apoptosis included cytoplasmic and nuclear shrinkage, chromatin condensation, and cytoplasmic blebbing with maintenance of the integrity of the cell membrane. As mentioned under *Results*, we also noted a distinct subpopulation of cells, termed provisionally "atypical" (Williams et al., 2001); they exhibit diminished or no detectable DNA while they maintain the basic cellular structure. These cells are morphologically different from classical apoptotic cells.

Transmission Electron Microscopy. Control MIA PaCa-2 cells or those treated with NSAIDs or NO-NSAIDs for 48 h were gently washed with serum-free medium and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). These cells were scraped and pelleted by centrifuging them at 10,000*g* for 5 min. After treatment with 1% osmium tetroxide, the block stained cells were dehydrated in graded ethanol, infiltrated with propylene oxide, and embedded with EMBED (Electron Microscopy Science, Fort Washington, PA) overnight and cured at 60°C for 48 h. Silver sections were cut with an Ultracut microtome, collected on a formvar and carbon-coated grid, stained with uranyl acetate and Reynolds' lead citrate, and viewed on a JOEL 100 CX II electron microscope (Tokyo, Japan).

Western Blotting. We followed standard protocols for protein extraction and quantification (Park et al., 1995). Proteins were separated on 10% polyacrylamide gel electrophoresis gel and electroplated on polyvinylidene difluoride membranes. After blocking membranes in 5% nonfat dry milk, they were incubated with antibodies to COX-1 or -2 for 1 h. Membranes were washed and incubated with secondary horseradish peroxidase-linked secondary antibody and developed by the enhanced chemiluminescence system. Antibodies against COX-1 and COX-2 and positive control standards were from Santa Cruz Biotechnology.

Statistics. Data are presented as means ± S.E.M. for at least three different sets of plates and treatment groups. Statistical comparison among the groups was performed using a one-way analysis of variance followed by the least significant difference method.

Results

Effect of NO-NSAIDs on a Pancreatic Cancer Cell Line

NO-NSAIDs Inhibit Pancreatic Cancer Cell Growth. We studied the effect of NO-aspirin and aspirin, NO-sulindac and sulindac, and NO-indomethacin and indomethacin on cell growth. MIA PaCa-2 cells exposed to various concentrations of these compounds were seeded in six-well plates at a density of 25×10^3 cells/cm^2 and harvested every 24 h for 72 h. From their growth curves, IC_{50} values were calculated (Fig. 2; Table 1).

All three NO-NSAIDs were very effective in inhibiting the growth of these pancreatic cancer cells. NO-ASA had the lowest IC_{50} values, their average value ranging between 7 and 22 μM over the 72 h of study. The corresponding IC_{50} values for NO-sulindac and NO-indomethacin were 60 to 92 and 48 to 82 μM, respectively. Thus, the potency NO-sulin-

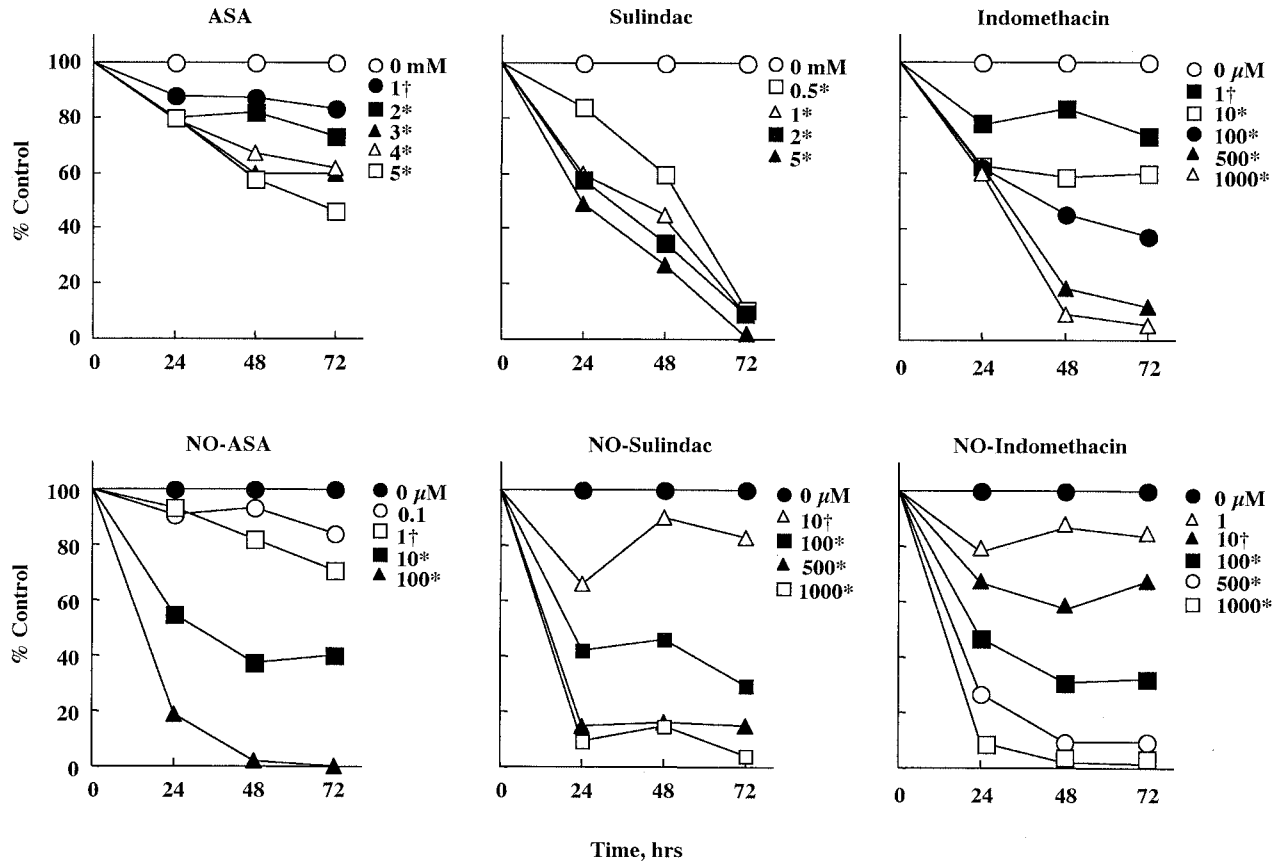

Fig. 2. Effect of NO-aspirin, NO-sulindac, and NO-indomethacin on MIA PaCa-2 cell growth. Cells were treated with various concentrations of NO-aspirin, NO-sulindac, or NO-indomethacin and their corresponding traditional NSAIDs, as described under *Materials and Methods*. Cell numbers were determined at 24, 48, and 72 h. Results represent the mean of at least three different experiments with duplicate plates. S.E.M. bars are not included for clarity. †, $P < 0.05$; *, $P < 0.01$ compared with untreated cells at all time points. See Table 1 for IC_{50} values ± S.E.M.

TABLE 1

IC_{50} values of NSAIDs and NO-NSAIDs in MIA PaCa-2 cells

Cells were treated with various concentrations of NO-aspirin, NO-sulindac, and NO-indomethacin and their corresponding traditional NSAIDs, as described under *Materials and Methods*. Cell numbers were determined at 24, 48, and 72 h from which IC_{50} values were calculated. Results are mean ± S.E.M. of three to seven different experiments done in duplicate.

Compound	IC_{50}		
	24 h	48 h	72 h
		μM	
Aspirin	>5000†	>5000†	4800 ± 200
NO-aspirin (NCX4040)	22 ± 2*	7.5 ± 1*	7.0 ± 1*
Ratio	>227	>588	695 ± 30
Sulindac	4600 ± 150	800 ± 50	320 ± 15
NO-sulindac (NCX1102)	92 ± 5*	65 ± 4*	60 ± 3*
Ratio	50 ± 1	12 ± 0.4	5 ± 0.1
Indomethacin	>1000†	440 ± 40	170 ± 25
NO-indomethacin (NCX 2121)	82 ± 4*	50 ± 3*	48 ± 3*
Ratio	>12	9 ± 0.3	4 ± 0.2

* $P < 0.001$ compared to the corresponding traditional NSAID.
† Exceeded the maximum concentrations used in these studies.

dac and NO-indomethacin was 7- to 12-fold lower than that of NO-ASA.

All three NO-NSAIDs were significantly more potent in inhibiting the growth of these cells compared with their traditional counterparts, and among the three NO-NSAIDs, NO-ASA was the most potent, followed by NO-sulindac and then NO-indomethacin. Of the traditional NSAIDs, indomethacin was more potent than sulindac, which in turn was more potent than ASA in inhibiting the growth of these cells. The ratio of IC_{50} values (traditional NSAID/NO-NSAID) was highest for NO-ASA, indicating it to be >230-fold more potent than ASA, although the only enhancement that could be calculated accurately was for the 72 h time point (693-fold). The corresponding ratios for both NO-sulindac and NO-indomethacin ranged between 4 and 50, indicating that the chemical modification of the parent molecules imparts on them a differential enhancement in potency. Thus, from being the weakest inhibitor of MIA PaCa-2 cell growth has, as NO-ASA, become by far the strongest.

The inhibitory effect of the NO-NSAIDs on MIA PaCa-2 cell growth was accompanied by profound changes in their morphology as assessed by light microscopy. Figure 3, series 1, shows light microscopy of cells exposed for 48 h to either ASA or NO-ASA. It is evident that, compared with control cells, NO-ASA had a profound effect on cell density and cell morphology. Cells were shrunken, rounded, and with compromised anchorage to the culture plate. ASA at much higher concentrations failed to affect the morphology of these cells.

NO-NSAIDs Alter pancreatic Cancer Cell Kinetics. To evaluate the mechanism(s) involved in the reductions of cell growth, we analyzed the effect of each of these compounds on cell renewal and cell death, two determinants of cell growth. Thus, we evaluated cell proliferation by measuring PCNA expression and cell death by morphological assessment of DAPI-stained cells following exposure to these compounds and also by determining the subdiploid peak in DNA content histograms. In addition, we determined the effect of these compounds on the distribution of MIA PaCa-2 cells in the various phases of the cell cycle.

Cell proliferation. Treatment of MIA PaCa-2 cells with NO-NSAIDs was accompanied by a significant antiproliferative effect. Figure 4 illustrates the results obtained with NO-sulindac, which induced a time- and concentration-dependent reduction in the expression of PCNA by these cells. The highest inhibition was obtained with the highest concentration at 72 h (27% compared with control, $P < 0.001$). PCNA expression was also significantly reduced by NO-ASA. At 48 h, we noted a concentration-dependent reduction as follows: 0.1 μM NO-ASA, 70 ± 2% of control; 1 μM, 62 ± 4% of control; 10 μM, 60 ± 4% of control; and with 50 μM, 53 ± 3% of control. Of note, the reduction of PCNA expression was not linearly related to reductions in cell number, suggesting that the participation of other kinetic effects, such as apoptosis, may play a role.

Cell death. Treatment of MIA PaCa-2 cells with NO-NSAIDs led to three morphologically distinct populations of cells on DAPI-stained samples: cells without morphological changes, which we termed unchanged, apoptotic cells, and atypical cells, as defined under *Materials and Methods*. As shown in Fig. 3 (series 2 and 3), NO-ASA induced significant rates of cell death. At 24 h, NO-ASA induced exclusively apoptotic cells in a concentration-dependent manner, reaching 65% at 100 μM. In contrast, there was negligible induction of atypical cells. ASA, at millimolar concentrations, also induced similar changes but of somewhat lesser magnitude (43% apoptotic cells). At 48 h, under the influence of NO-ASA, the apoptotic cells were more prominent, becoming the dominant cell type (89%) at 100 μM. At 72 h, 100 μM of NO-ASA, the highest concentration studied, induced a significant population of atypical cells (51%). The proportion of apoptotic cells was concentration-dependent and reached 49% at 100 μM NO-ASA. Traditional ASA failed to induce atypical cells above 3% even after treatment of the MIA PaCa-2 cells at 5 mM for 72 h.

The induction of apoptotic cells by NO-ASA was also documented by determining cell DNA content (detailed data not shown). At 48 h, following treatment with 10 μM NO-ASA, the subdiploid peak was 29%, and with 100 μM, it was 32%. These findings are consistent with those of the morphological study (DAPI). In contrast, ASA produced no discernible subdiploid peak; the sub-G_0/G_1 amount of DNA was less than 3.6% at ASA concentrations up to 5 mM. These findings are consistent with previous observations on the induction of cell death by ASA (Qiao et al., 1998a).

A similar study of cell death was performed on MIA PaCa-2 cells treated with NO-sulindac or sulindac for up to 72 h. Significant rates of apoptosis were noted when apoptosis was assessed by morphological criteria (DAPI staining; detailed data not shown). For example, treatment of these cells with NO-sulindac for 24 h induced concentration-dependent changes; apoptotic cells reached 48.3% at 1000 μM, whereas the atypical cells were 1.7%. At 48 h, 10 μM NO-sulindac induced 33% apoptotic and 1% atypical cells, whereas 100 μM NO-sulindac induced 51% apoptotic and 1% atypical cells. At 72 h, apoptotic cells approached 50%, and the atypical cells never exceeded 3%. Sulindac, studied in parallel at concentrations up to 5 mM, induced apoptotic cells in a concentration-dependent manner as well, reaching a maximum of 40% at 72 h in response to 5 mM sulindac. When the effect

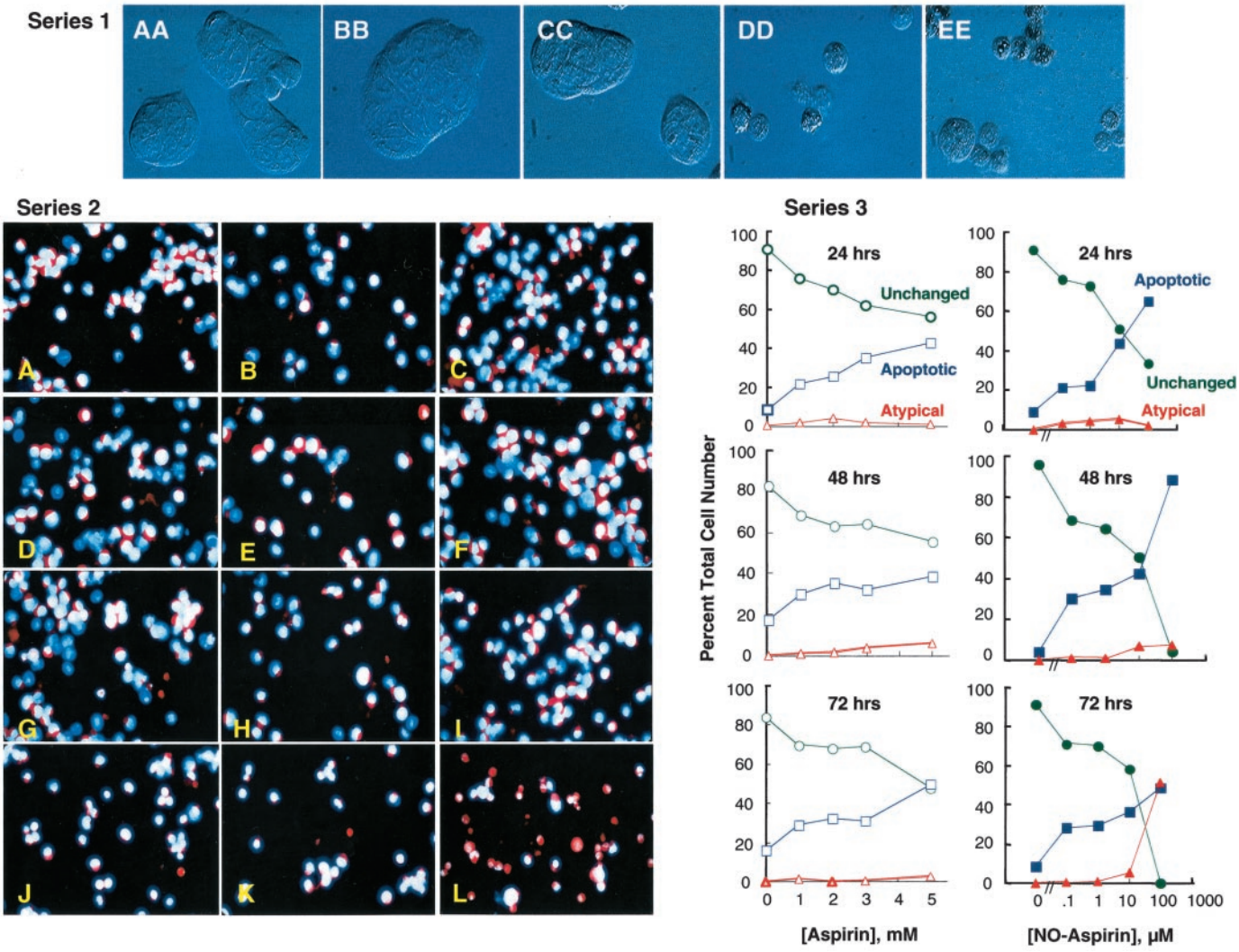

Fig. 3. Effect of NO-aspirin on morphology and apoptosis in MIA PaCa-2 cells. Series 1, light microscopy of control cells (no drugs, panel AA), treated with 1000 μM ASA (panel BB) and 5000 μM ASA (panel CC), and treated with 50 μM NO-ASA and 500 μM NO-ASA (panels DD and EE, respectively); all samples were exposed for 48 h. Series 2, control cells (untreated) at 24, 48, and 72 h (A–C), treated with 5000 μM ASA for 24, 48, and 72 h (D–F), treated with 1 μM NO-ASA for 24, 48 and 72 h (G–I), and treated with 100 μM NO-ASA for 24, 48 and 72 h (J–L). Series 3, using photographs similar to those shown in series 2, the cells in each population (unchanged, apoptotic, and atypical) were counted. The results are averages of two different experiments performed in duplicates.

of equimolar concentrations was compared, e.g., 500 μM of each compound, the percentage of apoptotic cells was 26 and 44% for sulindac and NO-sulindac, respectively.

The induction of apoptotic cells by NO-sulindac was also documented by determining cell DNA content (Table 2). For example, 48-h treatment with 200 μM NO-sulindac led to 12% apoptotic cells, whereas 1000 μM sulindac caused 16% apoptotic cells. These findings reflect the lower sensitivity of DNA content determinations in assessing apoptosis (Qiao et al., 1998a).

Cell cycle. NO-NSAIDs block cell cycle transitions of MIA PaCa-2 cells. For example, as shown in Table 2, following 48 and 72 h of treatment, NO-sulindac induced a block of the G_2/M to G_0/G_1 transition. This was evident by the increased percentage of cells in the G_2/M phase that was accompanied by corresponding reductions of the proportion of cells in S and G_0/G_1 phases. This effect was concentration-dependent.

NO-ASA induced a concentration-dependent block of the S to G_2/M transition (Fig. 5). Following 48 h of treatment with 10 μM NO-ASA, G_0/G_1 changed from 70% to 65% and S from 18 to 21%, whereas G_2/M did not change appreciably. These changes became more pronounced at 50 μM (G_0/G_1 from 70 to 54% and S from 18 to 30%, whereas G_2/M did not change). NO-ASA at 100 μM showed essentially the same effect as that at 50 μM, indicating a plateau of the effect.

NO-NSAIDs Induce Morphological Changes in Pancreatic Cancer Cells. Both the light microscopic findings and DAPI-stained cells make it clear that treatment of MIA PaCa-2 cells with NO-NSAIDs leads to significant morphological changes. We have studied these changes in greater detail by transmission electron microscopy. Electron micrographs of cells exposed to NO-ASA, NO-sulindac, or their traditional counterparts for 48 h highlight the dramatic effect of these NO-NSAIDs on pancreatic cancer cells (Fig. 6). Sulindac (1 mM) induces features of apoptosis with nuclear condensation and condensation and fragmentation, whereas 5 mM ASA induces similar but less pronounced changes, as previously reported for HT-29 cells (Qiao et al., 1998a). NO-

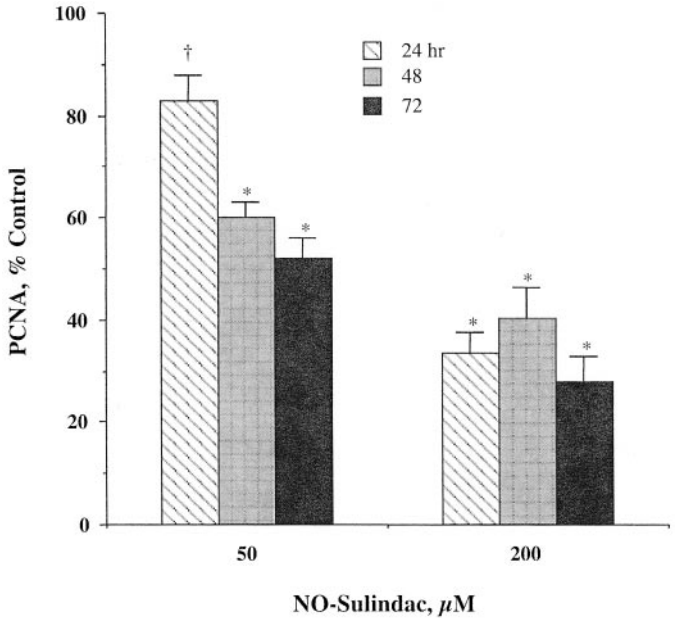

Fig. 4. The effect of NO-sulindac on MIA PaCa-2 cell proliferation. Cells were treated with 50 μM or 200 μM NO-sulindac and harvested at 24, 48, or 72 h. PCNA expression was determined as described under *Materials and Methods*. Results are the mean ± S.E.M. of three different experiments. †, $P < 0.05$; ∗, $P < 0.01$ compared with control.

TABLE 2

The effect of No-sulindac on cell cycle and apoptosis of MIA PaCa-2 cells

Pancreatic MIA PaCa-2 cells were treated with NO-sulindac and harvested at 24, 48, or 72 h. The percentage of cells in each phase was determined by flow cytometry, as described under *Materials and Methods*. Results are mean ± S.E.M. of three different experiments.

	Time	G_0/G_1	S	G_2/M	Apoptosis
	h				%
Control	24	40 ± 0.3	26 ± 0.7	32 ± 0.3	1.5 ± 0.4
	48	59 ± 0.4	19 ± 0.3	20 ± 0.6	1.3 ± 0.5
	72	74 ± 0.3	13 ± 0.4	10 ± 0.5	2.7 ± 0.4
100 μM	24	58 ± 0.7	17 ± 0.3	14 ± 0.3	9.5 ± 0.8
	48	41 ± 0.8	24 ± 1.0	26 ± 0.4	9.5 ± 0.8
	72	31 ± 0.2	26 ± 1.2	25 ± 1.1	16 ± 0.3
200 μM	24	63 ± 1.5	11 ± 1.1	18 ± 0.9	8.1 ± 1.1
	48	38 ± 3.2	22 ± 0.2	26 ± 0.2	12 ± 1.7
	72	33 ± 1.2	27 ± 0.3	23 ± 0.4	15 ± 0.6
400 μM	24	62 ± 0.9	10 ± 0.7	17 ± 0.9	10 ± 0.6
	48	38 ± 1.3	24 ± 2.3	26 ± 2.2	12 ± 1.0
	72	33 ± 1.4	28 ± 0.9	25 ± 1.3	13 ± 0.9
600 μM	24	62 ± 1.2	8 ± 0.8	22 ± 0.3	7 ± 0.7
	48	40 ± 1.3	24 ± 0.6	20 ± 1.0	15 ± 0.4
	72	32 ± 0.7	32 ± 0.8	24 ± 1.2	10 ± 0.7
1000 μM	24	62 ± 0.2	8 ± 0.6	21 ± 0.8	8 ± 0.4
	48	53 ± 0.9	13 ± 1.6	18 ± 0.6	16 ± 2.2
	72	37 ± 3.3	21 ± 1.2	18 ± 0.8	24 ± 3.2

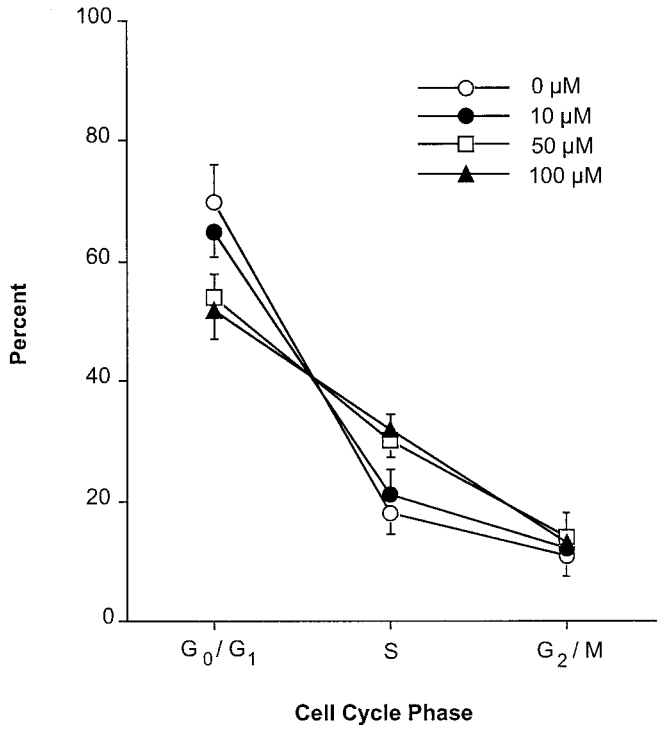

Fig. 5. Effect of NO-aspirin on the cell cycle in MIA PaCa-2 cells. Cells were treated for 48 h with various concentrations of NO-ASA, and their cell cycle phase distribution was determined by flow cytometry, as described under *Materials and Methods*. Results are the mean ± range for two independent studies done in duplicate.

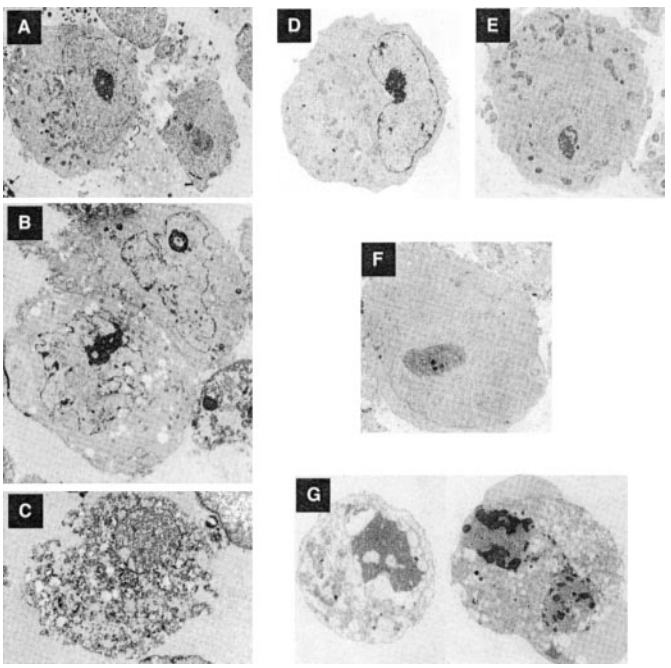

Fig. 6. Electron micrographs of NO-aspirin- and NO-sulindac-treated MIA PaCa-2 cells. Cells treated for 48 h were processed as described under *Materials and Methods*. A and D, control (no drugs); B, 1 mM sulindac; C, 1 mM NO-sulindac; E, 1000 μM ASA; F, 5000 μM ASA; G, 100 μM NO-ASA. Magnification: 1000× (A–C) and 1600× (D–G).

sulindac, at the same concentration, practically destroyed the cell, with extensive vacuolization of the cytoplasm, mitochondrial damage, and loss of the integrity of cell membrane. In addition, the nucleus is greatly damaged with loss of volume and texture, consistent with the features of the atypical cells on DAPI staining (Fig. 3, series 2). These changes are strongly suggestive of cell necrosis. NO-ASA also induced similar changes as NO-sulindac, however at much lower concentrations (Fig. 6, G).

COX Independence. COX represents the best-known mechanistic target of NSAIDs. Whether inhibition of COX by NSAIDs or NO-NSAIDs is required for their effects on cancer cell growth is debatable (Rigas and Shiff, 2000; Williams et al., 2001). To evaluate this question in our system, we assessed the effect of NO-ASA on the BxPC-3 pancreatic cancer

cell line, which, in contrast to MIA PaCa-2 cells, does express both COX isoforms (Fig. 7; Molina et al., 1999). BxPC-3 cells responded to NO-ASA in a similar manner as MIA PaCa-2 cells. At 48 h, the IC_{50} values for BxPC-3 cells were as follows: ASA = 4800 ± 150 μM and NO-ASA = 5.2 ± 0.7 μM. These values are very close to those obtained for MIA PaCa-2 cells subjected to the same experimental treatment (Fig. 7). The distribution of cell populations (unchanged, apoptotic, and atypical) was similar to that observed for MIA PaCa-2 cells (data not shown). These findings indicate that the presence of the COX enzymes likely did not play a significant role in the response of these cells to NO-ASA. The data from both cell lines in Fig. 7 show that NO-ASA is not only more potent than ASA but also more efficacious if we consider 100% inhibition of cell growth as our endpoint.

Effect of NO-NSAIDs on Other Cancer Cell Lines

We evaluated the effects of NO-ASA, NO-sulindac, and NO-ibuprofen on prostate, lung, colon, and tongue cancer cell lines. Following a 48-h treatment of these cell lines, all three NO-NSAIDs very effectively inhibited the growth of these cell lines (Fig. 8; Table 3). NO-ASA showed similar potency in

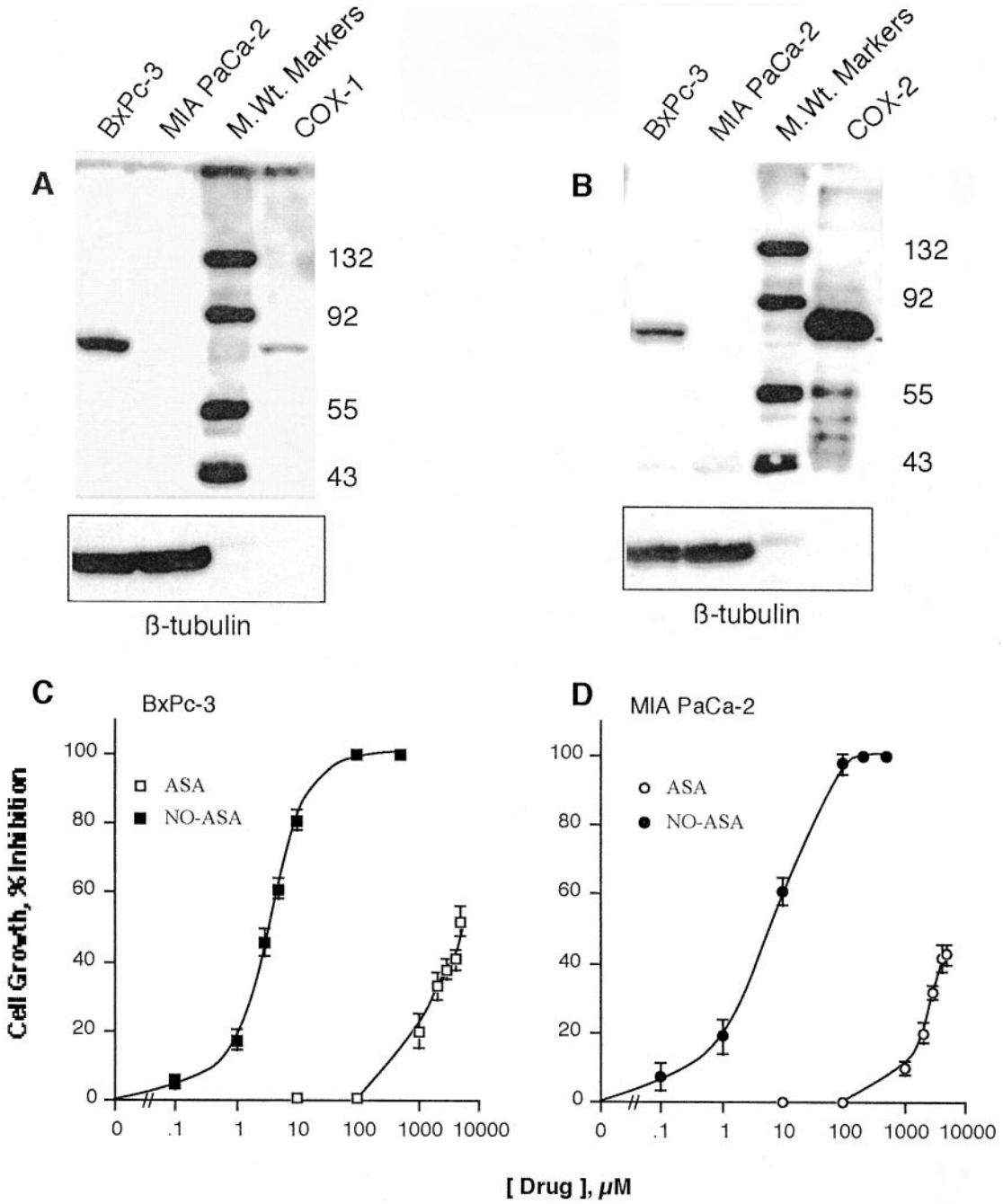

Fig. 7. Expression of COX-1 and COX-2 in MIA PaCa-2 and BxPC-3 pancreatic cells. Western blot analysis was carried out as described under *Materials and Methods*. A and B, show expression of COX-1 and COX-2 in untreated MIA PaCa-2 and BxPC-3 cells, respectively. C and D, show dose-response inhibition of cell growth in BxPC-3 and MIA PaCa-2 cells, respectively, after 48 h of treatment with ASA or NO-ASA. Results are the means ± S.E.M. for at least three different experiments done in duplicate.

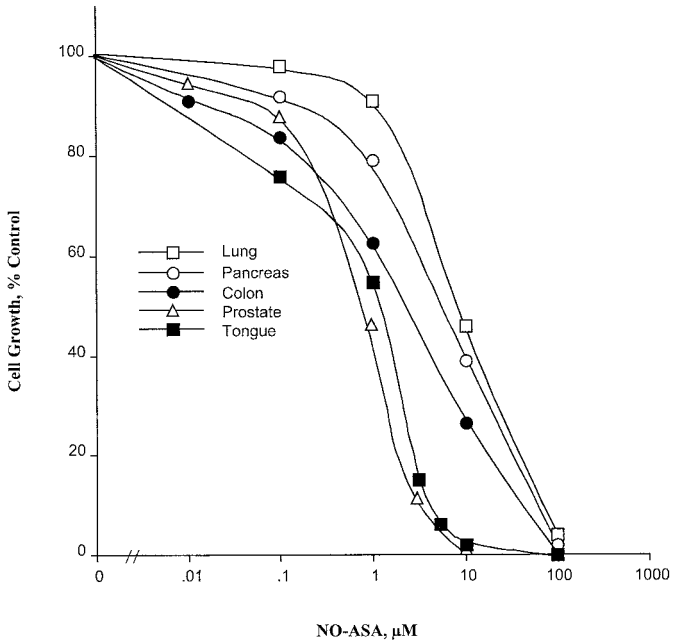

Fig. 8. Effect of NO-ASA on various cancer cell lines. Lung, pancreas (MIA PaCa-2), colon, prostate, and tongue cancer cell lines were treated with various concentrations of ASA and NO-ASA for 48 h, as described under *Materials and Methods*. Results are the means of at least three different experiments done in duplicate. S.E.M. bars are not included for clarity. See Table 3 for IC_{50} values ± S.E.M.

the growth inhibition of the prostate, colon, and tongue cell lines (IC_{50} between 0.8 and 2 μM), whereas its effect on lung cells was distinctly weaker (IC_{50}, 80 μM). NO-sulindac was most potent against colon cancer cells (IC_{50}, 28 μM); its IC_{50} values for these four cell lines varied about 3-fold (28–92 μM). Finally, NO-ibuprofen was most effective against lung cancer cells (IC_{50}, 35 μM), although its IC_{50} varied the least for the various cell lines (35–79 μM).

In all instances, NO-NSAIDs inhibited the growth of these cell lines more effectively than their traditional NSAID counterparts. In general, NO-ASA was 1 to 2 orders of magnitude more potent than NO-sulindac or NO-indomethacin. Compared with traditional ASA, its potency was significantly enhanced. Even though NO-ASA was 50-fold more potent than ASA in inhibiting the growth of lung cancer cells, its IC_{50} was even more remarkably reduced compared with ASA for the other five cell lines, being >2000 to 6000 times lower. Compared with sulindac, NO-sulindac showed its weakest effect in lung cancer cells, being close to that of traditional sulindac, whereas it was 16 to >29 times more potent in inhibiting the growth of the other cancer cell lines. NO-ibuprofen was probably most effective against lung cancer cells, although in four of five cell lines, it was not possible to determine accurately the IC_{50} of traditional ibuprofen because it was never reached under our experimental conditions. It is apparent that these NO-NSAIDs did not parallel each other in terms of their potency in inhibiting the growth of each of these cell lines, indicating both quantitative and qualitative differences in their effects on cancer cell lines and providing a type of tissue specificity.

Discussion

Our results establish that several NO-NSAIDs inhibit the growth of cancer cell lines arising from a variety of human tissues. In addition, NO-NSAIDs are more potent than their traditional NSAID counterparts, with their potency being 11 to 6000-fold greater than that of their counterparts (except for the effect of sulindac on lung cancer cells). Of the four NO-NSAIDs that we studied, NO-ASA was consistently the most potent NO-NSAID in all cell lines tested (except for the lung cancer cell line), sometimes in excess of 100-fold over the other three NO-NSAIDs.

These findings have several important features. Our data establish that the tissue of origin of the cancer cell line does not restrict the growth inhibitory effect of NO-NSAIDs. In fact, our data provide evidence that both adenomatous and squamous cancer cell lines are susceptible to this effect of various NO-NSAIDs. Although only seven cell lines originating from five different tissues have been studied, we suspect that our findings are part of a generalized effect, especially since the growth of no cell line failed to be inhibited by NO-NSAIDs. Obviously, more work is needed to further substantiate the generalization of this property.

NO-NSAIDs achieve their growth inhibitory effect on cultured colon cancer cells through a complex effect on cell kinetics, involving cell renewal, cell death, and the cell cycle. The results from the pancreatic cells illustrate this point. For example, 48-h treatment of these cells with 50 μM NO-sulindac (a concentration close to its IC_{50}) inhibited PCNA

TABLE 3

IC_{50} values of NSAIDs and NO-NSAIDs in different cell lines

Lung, pancreas (MIA PaCa-2), colon, prostate, and tongue cancer cell lines were treated with various concentrations of NO-aspirin, NO-sulindac, and NO-ibuprofen and their corresponding traditional NSAIDs, as described under *Materials and Methods*. Cell numbers were determined at 48 h from which IC_{50} values were calculated. Results are mean ± S.E.M. of three to seven different experiments done in duplicate.

Compound	IC_{50} at 48 h				
	Prostate	Lung	Colon	Tongue	Pancreas
			μM		
Aspirin	4820 ± 180	4090 ± 150	> 5000†	> 5000†	> 5000†
NO-aspirin (NCX4040)	0.8 ± 0.2*	80 ± 5*	2 ± 0.5*	1.5 ± 0.3*	7.5 ± 1*
Ratio	6200 ± 470	51 ± 1	> 2000	> 2777	> 588
Sulindac	1450 ± 100	75 ± 5	650 ± 50	> 1000†	800 ± 50
NO-sulindac (NCX1102)	92 ± 7*	65 ± 3*	28 ± 4*	35 ± 5*	65 ± 4*
Ratio	16 ± 0.4	1 ± 0.1	23 ± 1	> 25	12 ± 0.3
Ibuprofen	> 1000†	> 1000†	> 1000†	> 1000†	440 ± 30
NO-ibuprofen (NCX2111)	45 ± 4*	35 ± 3*	50 ± 4*	79 ± 6*	40 ± 4*
Ratio	> 20	> 26	> 19	> 12	11 ± 0.4

* $P < 0.001$ compared to the corresponding traditional NSAID.
† Exceeded the maximum concentrations used in these studies.

expression by about 40%. Following treatment with 100 μM NO-sulindac for 48 h, slightly over half of the cells were apoptotic. Another contributor to their growth inhibitory effect may be the block on cell cycle phase transitions induced by these compounds, such as the G_2/M to G_0/G_1 block that we noted in pancreatic cells. Traditional NSAIDs are known to profoundly affect cell cycle transitions through changes in proteins that control them (Shiff et al., 1995; Qiao et al., 1998b; Rigas and Shiff, 1999). It is thus conceivable that NO-NSAIDs modulate the expression and/or function of molecules that constitute the so-called cell cycle machinery. Gansauge et al. (1998) showed that endogenous NO in MIA PaCa-2 cells induced apoptosis, G_1 arrest, and increased the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$. NO-NSAIDs, bearing NSAID and NO-donating moieties, represent a more complex situation. This is especially true since the same authors demonstrated in fibroblasts that exogenous NO donors increased cell proliferation and the S/G_2 fraction, whereas endogenous NO inhibited cell proliferation and led to G_1 arrest (Gansauge et al., 1997).

Although it is difficult to assess the contribution of these changes to the final cell number, it appears that both the antiproliferative and proapoptotic effects of NO-sulindac make an important contribution to its cell growth inhibitory effect, with perhaps the cell death effect predominating. The overall contribution of the cell cycle block is difficult to quantify.

It is interesting that only NO-ASA induced the so-called atypical cells and only at the highest concentration studied (100 μM for 72 h). The contribution of these cells to the overall cell growth inhibitory effect of NO-ASA is unclear. The presence of these cells appears to be a distinguishing feature of this NO-NSAID, which is clearly the most potent of all. It is conceivable that somehow NO released by NO-ASA may convert apoptosis into necrosis or some variant thereof (Melino et al., 2000).

The potency of an NSAID in inhibiting cell growth does not predict the potency of the corresponding NO-NSAID. ASA is the weakest of the three traditional NSAIDs in inhibiting the growth of any of the cell lines. Yet, NO-ASA had the highest potency of all, evidenced by its very low IC_{50} values. The magnitude of this change is reflected in the very large IC_{50} ratios of ASA/NO-ASA that exceed those of the other NO-NSAIDs, at times by over 100-fold. The process of nitration of an NSAID may impart an array of properties onto the new molecule, some of which affect cell growth. That nitration of different NSAIDs does not lead to the same cell growth inhibition, however, underscores how complex their effect on cell growth is. The fact that the spacer molecule is not the same in all the NO-NSAIDs used in this study (Fig. 1) makes any efforts to detect an underlying common mechanism even more difficult.

There was no discernible pattern in the response of the various cell lines to any of the NO-NSAIDs used in our study. Although NO-ASA had, in most instances, the lowest IC_{50} of all NO-NSAIDs, this was not true for the lung cancer cell line. Extreme variability was observed in the response of all cell lines to the other NO-NSAIDs.

In the last few years, COX-2 overexpression has been considered to play an important role in carcinogenesis (Eberhart et al., 1994). For example COX-2 is overexpressed at various stages of several tumors, including those of colon (Prescott and Fitzpatrick, 2000), lung (Hasturk et al., 2002), and pancreas (Molina et al., 1999). Inhibition of COX enzymes, the best-known pharmacological target of NSAIDs, may account for the chemopreventive properties of NSAIDs and perhaps NO-NSAIDs. Our data show that a pancreatic cell line that expresses COX enzymes and one that does not respond similarly to NO-NSAIDs. This indicates that an effect on COX may not be required for the effect of NO-NSAIDs, in agreement with similar observations by others and us using traditional NSAIDs (Hanif et al., 1995). It is, therefore, possible that the effect on COX is part of a mechanistic redundancy of NSAIDs, and perhaps NO-NSAIDs, that ensures their remarkable effect on cancer (Rigas and Shiff, 2000). Consistent with this notion is also the recent observation that NSAIDs increase the expression of an NSAID-activated gene (NAG-1), which has proapoptotic and antitumorigenic activity in cells devoid of COX activity (Baek et al., 2001, 2002). This observation is important since it may contribute to our understanding of how cells devoid of COX expression still respond to NSAIDs in terms of growth inhibition. The effect of NO-NSAIDs on expression of NAG-1 in various cell lines is currently under investigation in our laboratory. Taken together, our data make it clear that various NO-NSAIDs significantly inhibit the growth of cancer cell lines originating from various epithelial tissues. The effect of NO-NSAIDs was variable but always enhanced compared with traditional NSAIDs. This variability concerned both the type of NO-NSAID (i.e., the same NO-NSAID had a variable result in the various cell lines) and the type of cell line (i.e., a given cell line responded differently to the various NO-NSAIDs).

A minimalist interpretation of these data is that the various NO-NSAIDs share a property that affects a process common to all cancer cell lines, which can be surmised to be a fundamental one. A reasonable candidate for the former is the $-NO_2$ group on the NO-NSAIDs; inspection of the structures of all NO-NSAIDs used in this study (Fig. 1) indicates that this group is the only shared feature among them. An alternative or complementary idea would be that another shared property of these molecules is the "NSAID component" of all of them, which expresses a general pharmacological function and not a structural feature. Needless to say, this property cannot account for their enhanced potency. It is more difficult to speculate on the shared property of the cancer cell lines due to the higher level of complexity of the cancer cell as opposed to that of the NO-NSAID molecule. Nevertheless, the two fundamental contributors to cell growth are cell renewal and cell death. Since the induction of cell death appears to be a more prominent effect of NO-NSAIDs on these cell lines than inhibition of proliferation, one might consider that the common property targeted by these NO-NSAIDs is related to cell death. It is thus conceivable that the NO liberated by these compounds activates or enhances cell death against a background of the effect of the NSAID part of each molecule.

In this regard, the following are important considerations: 1) determination of the effect of NO alone and of the aromatic ring in the spacer molecule (we are currently evaluating both by using denitrated analogs of NO-NSAIDs and analogs bearing an aliphatic spacer molecule) and 2) differentiation of downstream mediators of the apoptotic and cell cycle effects to assess whether NO-NSAIDs recruit alternative pathways to augment the effect of the traditional NSAID.

It is interesting to speculate on the potential usefulness of NO-NSAIDs for cancer treatment or prevention. Certainly, it is not always safe to extrapolate cell culture findings into intact animals, including humans. Nevertheless, when our data are viewed against the background of the chemopreventive effect of traditional NSAIDs in a variety of human tumors, mostly of the digestive system (Thun et al., 2002), it may not be too outlandish to predict that these compounds could be useful at least in the prevention of one or more of these tumors. The present data combined with recent evidence of their safety when administered to humans make a compelling argument for their further preclinical evaluation in animal models of cancer and for further elucidation of their molecular mechanism of action.

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