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Nitric Oxide-releasing Nonsteroidal Anti-inflammatory Drugs (NSAIDs) Alter the Kinetics of Human Colon Cancer Cell Lines More Effectively than Traditional NSAIDs: Implications for Colon Cancer Chemoprevention[1]

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Abstract

Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs), consisting of a known nonsteroidal anti-inflammatory drug (NSAID) and a nitric oxide (NO)-releasing group, are reported safer than NSAIDs. To assess their potential in colon cancer chemoprevention, we studied *in vitro* the effect of NO-aspirin, NO-sulindac, and NO-ibuprofen on colonocyte kinetics. These three NO-NSAIDs reduced the growth of cultured HT-29 colon adenocarcinoma cells much more effectively than the corresponding NSAIDs; *e.g.*, at 24 h, their IC_{50} values were as follows: (*a*) aspirin, >5000 μM; (*b*) NO-aspirin, 1 μM; (*c*) sulindac, 750 μM; (*d*) NO-sulindac, 150 μM; (*e*) ibuprofen, >1000 μM; and (*f*) NO-ibuprofen, 42 μM. This effect was due to inhibition of proliferation and induction of apoptosis and perhaps to the induction of novel cell changes, characterized by extensive DNA degradation. NO-NSAIDs also blocked the G_0-G_1 to S cell cycle transition. Their superior effectiveness compared with traditional NSAIDs, combined with their reported safety, makes them promising candidates for chemopreventive agents against colon cancer.

Introduction

Ample evidence supports the notion that chemoprevention has the potential to be a major component of the control of colorectal cancer, one of the most common human malignancies in the Western world (1). NSAIDs[3] are prototypical chemopreventive agents against colorectal cancer (reviewed in Refs. 2 and 3), but side effects are a major obstacle to their large-scale application to prevent colorectal cancer. NSAID toxicity includes (*a*) GI side effects, which range from dyspepsia to GI bleeding, obstruction, and perforation; (*b*) renal side effects, including a wide range of tubular, interstitial, glomerular, and vascular lesions; and (c) a large number of additional side effects, some of them serious, ranging from hypersensitivity reactions to the distinct salicylate intoxication (summarized in Ref. 4). Among patients using NSAIDs, up to 4% per year suffer serious GI complications; in 1998, the number of deaths in the United States from NSAID-induced GI complications was 16,550, virtually identical to the 16,685 deaths from AIDS (5, 6). The synthesis of NO-NSAIDs represents a novel approach to reduce the side effects of NSAIDs, especially their gastric toxicity (7–9). NO-NSAIDs consist of a known NSAID molecule and a NO-releasing group (typically $-NO_2$) linked to it via a chemical spacer (Fig. 1). The rationale for their development was based on the observation that NO possesses some of the same properties as PGs within the gastric mucosa. NO increases mucosal blood flow, mucous release, and repair of the mucosa, whereas it inhibits neutrophil activation and adherence. These effects can theoretically compensate for gastric PG reduction. Coupling a NO-releasing moiety to a NSAID might deliver NO to the site of NSAID-induced damage and thus decrease gastric toxicity. Existing data, mostly from animal studies, indicate that this prediction may be true (7–9).

Like all cancers, colon cancer reflects one or more disturbances in tissue homeostasis. Changes in the rates of colonocyte proliferation, apoptosis, or both participate in colonic tumorigenesis (2). Work by us and others has established that traditional NSAIDs exert a major effect on cell kinetics (*e.g.*, Ref. 10). *In vitro*, their effects include inhibition of cell proliferation, induction of apoptosis, and a significant block in cell cycle transitions leading to cell quiescence. There is evidence that at least some of these changes occur in animals and humans. To assess the chemopreventive potential of these novel compounds against colon cancer, we evaluated the effect of three NO-NSAIDs, namely, NO-aspirin, NO-sulindac, and NO-ibuprofen, on colonocyte kinetics. Their parent NSAIDs represent important structural classes of NSAIDs and have been studied extensively for their effect on colon cancer. This report presents our findings, which indicate that *in vitro* NO-NSAIDs are much more effective on colonocyte kinetics than their parent compounds.

Materials and Methods

Cell Lines. HT-29 and HCT-15 human colon adenocarcinoma cell lines (American Type Culture Collection, Manassas, VA) were grown as monolayers in McCoy 5A medium and RPMI 1640, respectively, and supplemented with 10% FCS (Mediatech, Herndon, VA), penicillin (50 units/ml), and streptomycin (50 μg/ml; Life Technologies, Inc., Grand Island, NY). Cells were seeded at a density of 1.5×10^6 cells/100-cm^2 culture dish 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}-1H-indene-3-acetic acid 4-(nitrooxy)butyl ester), NO-ibuprofen (NCX2210; *trans*-3-{4-[α-methyl-4-(2-methylpropyl)benzeneacetylpxy]-3-methoxyphenyl}-2-propenoic acid 4-nitrooxy)butyl ester), and NO-aspirin (NCX4040; 2-(acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester) were gifts of Dr. P. Del Soldato (Nicox, SA, France). Stock (100 mM) solutions of NO-NSAIDs and NSAIDs were prepared in DMSO (Fisher Scientific, Fair Lawn, NJ). All compounds were added to the culture medium immediately before plating. Final DMSO 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. For each subset, we analyzed ≥10,000 events. All parameters were collected in listmode files. Data were analyzed on an XL Elite

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; NO, nitric oxide; NO-NSAID, NO-releasing NSAID; DAPI, 4′,6-diamidino-2-phenylindole; GI, gastrointestinal; FBS, fetal bovine serum; PCNA, proliferating cell nuclear antigen; PG, prostaglandin; COX, cyclooxygenase; ASA, aspirin.

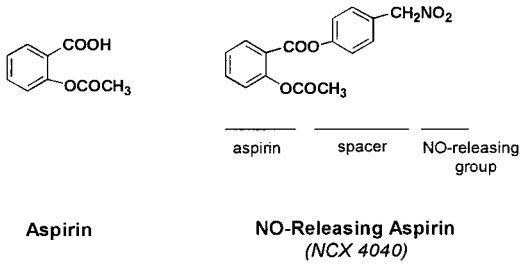

Fig. 1. The chemical structure of NO-aspirin. The three structural components of this novel derivative of aspirin (designated *NCX 4040*) are indicated: the spacer molecule links the traditional NSAID to $-NO_2$, which can release NO. The structure of traditional aspirin is also shown for comparison.

Table 1 *The IC_{50} of NO-NSAIDs and NSAIDs in HT-29 cells*

Compound	IC_{50} (μM)		
	24 h	48 h	72 h
Aspirin	>5000[a]	2540	2540
NO-aspirin (NCX 4040)	1	2	2
Ibuprofen	>1000	>1000	>1000
NO-ibuprofen (NCX 2210)	42	48	44
Sulindac	750	682	240
NO-sulindac (NCX 1102)	150	33	12

[a] Exceeded the maximum concentration used in these studies.

Work station (Coulter) 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 for 10 min at 4°C), resuspended, and incubated in PBS containing 1% FBS/0.5% NP40 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 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. Cells were then washed and incubated with goat antimouse 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 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 from 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 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.

Results

NO-NSAIDs Reduce the Number of Cultured Colon Cancer Cells More Effectively than Their Parent Compounds. Compared with their parent NSAIDs, all three NO-NSAIDs reduced the number of HT-29 cells in culture at lower concentrations and after shorter incubation periods (Table 1; Fig. 2). Remarkably, even at the maximal concentrations used, ASA and ibuprofen failed to reduce the number of cells by half, whereas their NO-derivatives reduced the cell number to near zero by 24 h. Indeed, the ratio of the IC_{50} for ASA compared with that of NO-ASA (ASA:NO-ASA) ranged between 1270 and >5000 over the 72 h of observation; for ibuprofen:NO-ibuprofen, it was >42, and for sulindac:NO-sulindac, it ranged between 5 and 20. Similar ratios were obtained with HCT-15 cells (data not shown).

NO-NSAIDs Inhibit Cell Proliferation. To evaluate the mechanism(s) involved in these substantial reductions in cell number, we determined the effect of NO-NSAIDs on cell proliferation by assaying the expression of PCNA. As shown in Table 2, all three compounds reduced the expression of PCNA in a concentration-dependent manner. The extent of this effect, however, differed substantially between these three compounds. For example, 48 h of exposure to 100 μM NO-ASA reduced PCNA expression by 40% and the cell number by 99%, compared with control. The corresponding values were 22% and 77% for NO-sulindac and 42% and 76% for NO-ibuprofen. Furthermore, the reduction in 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.

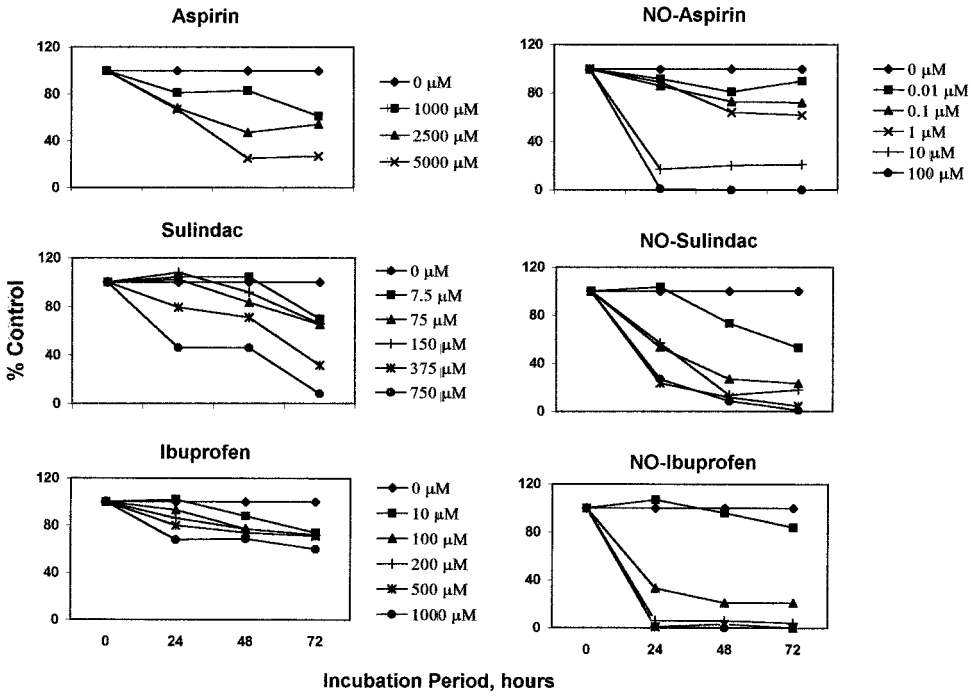

Fig. 2. The effect of NO-NSAIDs on HT-29 cell growth. HT-29 cells were treated with various concentrations of NO-NSAIDs or their corresponding traditional NSAIDs as described in "Materials and Methods." Cell number was determined at 24, 48, and 72 h.

Table 2 *The effect of NO-NSAIDs on HT-29 cell proliferation, apoptosis, and cell cycle*

Values are the average of duplicate plates. Studies were repeated at least once and were within 10%.

	Proliferation	Apoptosis		Cell cycle phases		
Concentration, (μM)	PCNA, % (+)	DAPI-stained apo/atypical (%)	sub-G_0-G_1	G_0-G_1	S	G_2-M
NO-aspirin						
48 h						
0	90.5	2.2/0.7	2.2	53.3	35.3	11.2
0.01	87.5	2.3/0.4	2.6	52.1	36.9	11.3
0.1	88	3.3/1.5	2.7	52.4	39.1	8.5
1	80	2.8/0.0	2.5	54.8	39.6	5.7
10	74.5	22.2/7.7	23.3	71.5	19.6	9.0
100	54.5	45.7/54.3	18.8	83.9	13.8	2.5
72 h						
0	92	3.5/1.8	3.9	64.8	21.0	8.7
0.01	90	3.2/1.6	4.3	70.0	21.9	8.2
0.1	96	1.1/1.0	4.0	71.2	20.1	8.7
1	88	3.1/0.0	4.4	70.8	21.1	8.2
10	69	10.9/9.6	18.1	61.9	25.3	10.8
100	43	42.0/58.0	18.4	87.4	10.0	2.6
NO-Ibuprofen						
48 h						
0	93	2.2/0.7	2.7	63.8	24.4	11.8
50	88	11.2/7.6	3.8	61.2	26.2	12.6
100	53	19.2/45.7	12.5	61.3	26.8	11.9
200	46	39.8/60.2	20.4	80.1	14.4	5.5
72 h						
0	89	3.5/1.8	3.4	62.8	24.3	12.9
50	87	4.0/1.7	2.2	59.4	26.7	13.9
100	61	28.7/41.0	7.9	67.9	24.0	8.2
200	57	34.0/58.8	17.9	88.4	9.3	2.4
NO-Sulindac						
48 h						
0	91	2.2/0.7	6.8	58.1	30.2	11.7
10	81	6.1/1.2	10.4	54.2	41.9	4.0
100	71	33.9/7.4	13.2	76.0	12.0	12.2
200	55	36.7/5.0	11.5	76.9	12.1	11.2
72 h						
0	92	3.2/1.9	6.2	66.7	21.7	11.6
10	84	3.1/1.2	6.2	68.0	20.5	11.6
100	55	40.2/13.4	10.0	77.5	12.6	10.0
200	51	36.9/10.4	13.3	65.6	24.7	9.8

NO-NSAIDs Induce Apoptosis. NO-NSAIDs induced apoptosis, which was detected by morphological evaluation of DAPI-stained cells and also by determination of cell DNA content. All three NO-NSAIDs induced apoptosis in a concentration-dependent manner (Table 2). The effect appeared maximal at 48 h.

Microscopic examination of DAPI-stained cells revealed that, compared with controls, cultures treated with NO-NSAIDs had an increased number of apoptotic cells, as defined by standard morphological criteria such as cytoplasmic and nuclear shrinkage or chromatin condensation (Table 2; Fig. 3). For example, exposure of cells to 100 μM NO-ASA for 48 h increased the frequency of apoptotic cells from 2% in controls to 46%; 1 μM NO-ASA had no such effect. The other two compounds had a similar but less pronounced effect on apoptosis: 48 h of exposure to 100 μM NO-ibuprofen resulted in 19% apoptotic cells, whereas NO-sulindac under identical culture conditions increased apoptosis to 34%.

An interesting phenomenon was the appearance of a new population of cells. These cells exhibited diminished or no detectable DNA. On the basis of morphological criteria, they were clearly different from apoptotic cells. Preliminary study of such cells indicates that the loss of DNA is a progressive event, which in some cells is probably accompanied by a reduction in cell size. These cells have been termed "atypical," and their frequency is listed in Table 2. Atypical cells were quite frequent, especially in cultures treated with higher concentrations of NO-ASA and NO-ibuprofen, where they constituted the absolute majority of all cellular entities. Indeed, in some instances, atypical and classical apoptotic cells were the only two cell types observed. It would not be unreasonable to hypothesize that the progressive loss of DNA adversely affects the longevity of these atypical cells. Thus, this phenomenon may contribute to cell death (or be a

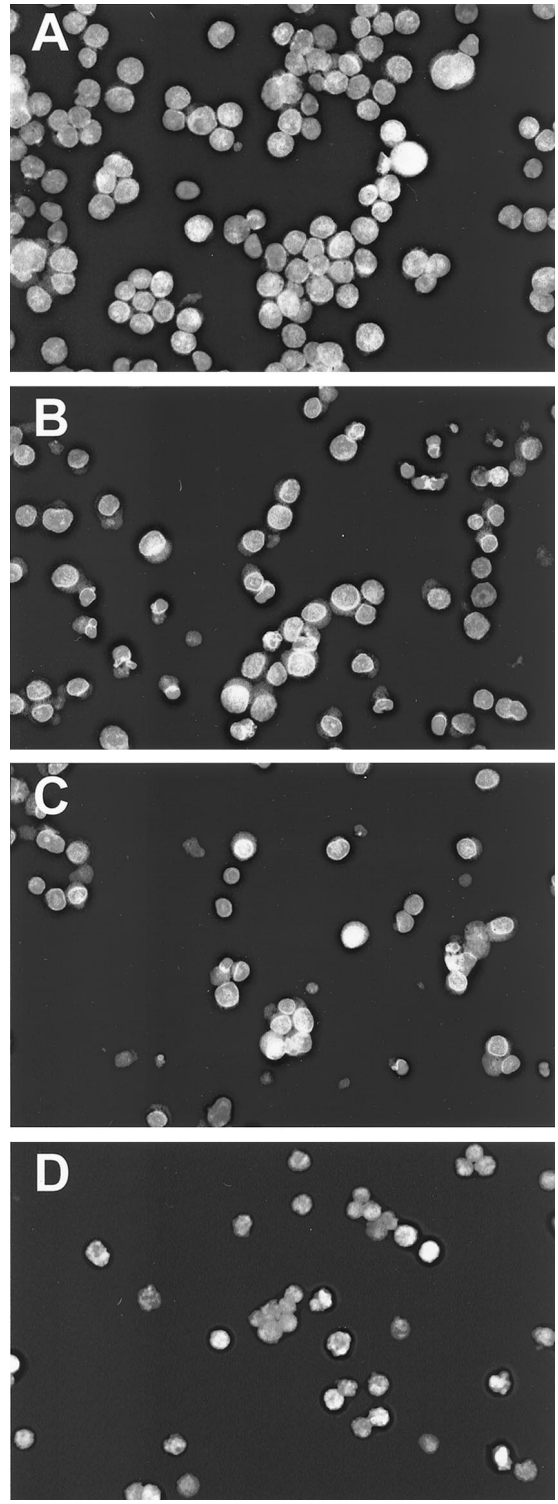

Fig. 3. The effect of NO-aspirin on apoptosis in HT-29 cells. *A*, control samples (no NO-NSAID treatment) showed rare apoptotic cells. Samples treated for 72 h with 200 μM NO-sulindac (*B*), 10 μM NO-aspirin (*C*), or 100 μM NO-ibuprofen (*D*) show classical apoptotic cells (cytoplasmic and nuclear shrinkage; chromatin condensation). In addition, especially in *D*, there are cells that maintain their basic structure but show diminished to no detectable amounts of DNA without DNA condensation or cytoplasmic shrinkage (atypical cells in Table 2).

consequence of it) and thereby to the diminished cell number observed after exposure to the various NO-NSAIDs.

As shown in Table 2, all three NO-NSAIDs also induced apoptosis in HT-29 cells when apoptosis was determined by assaying the proportion of cells with a subdiploid DNA content. For example, exposure to 10 μM NO-ASA resulted in 20% apoptotic cells. Indeed, at that concentration, the effect already appeared maximal, being around 10-fold higher than control. The relative efficacy of these compounds in inducing apoptosis can be assessed by determining the frequency of apoptotic cells after treatment of HT-29 cells with equimolar concentrations of these compounds for the same period of time. Thus, after exposure to 100 μM of each NO-NSAID for 48 h, the increase in the percentage of apoptotic cells over control was 8.7-fold for NO-ASA, 4.6-fold for NO-ibuprofen, and 1.9-fold for NO-sulindac. As expected (10, 11), the percentage of apoptotic cells was higher when apoptosis was determined by morphological criteria rather than by cell DNA content, although the changes in apoptosis detected by these two methods were always concordant.

NO-NSAIDs Block Cell Cycle Transitions. All three NO-NSAIDs significantly altered the cell cycle distribution of HT-29 cells, inducing a block in the G_0-G_1 to S transition. This was evident by the increased percentage of cells in the G_0-G_1 phase that was accompanied by corresponding reductions of the proportion of cells in S and G_2-M phases. This effect was concentration dependent. NO-ASA was the most effective of the three in inducing these changes. This is evident, for example, when equimolar concentrations of the three compounds are compared, *e.g.*, exposure to 100 μM for 48 h. NO-ASA increased the G_0-G_1 fraction by 57% over control *versus* no change or a 31% increase after exposure to NO-ibuprofen and NO-sulindac, respectively. The corresponding changes for the S-phase were a 61% reduction for NO-ASA, no change for NO-ibuprofen, and a 60% reduction for NO-sulindac. For G_2-M, there was a 78% reduction for NO-ASA and virtually no change for the other two.

Discussion

Our data demonstrate that three members of the family of the novel NO-NSAIDs inhibit cell growth much more effectively than their parent NSAIDs; their superior effectiveness reached a very high level in the case of NO-ASA.

NO-NSAIDs inhibited cell growth through an effect on cell proliferation, apoptosis, or both. Although it is difficult to accurately determine their individual contribution to these complex kinetic changes, our data provide some indication of the relative magnitude of these effects. The case of NO-ASA illustrates this point. Compared with controls, exposure to 1 μM NO-ASA for 48 h reduced the cell number by 11% and PCNA expression by 17%, whereas there was no detectable induction of apoptosis and no change in the cell cycle phase distribution of these cells. Thus, it appears that at this concentration of NO-ASA, there is only a minor antiproliferative effect. In contrast, exposure of colonic tumor cells to 10 μM NO-ASA reduced the number of cells by 83% and PCNA expression by 25% and increased apoptosis at least 10-fold, not taking into account the atypical cells. This treatment also increased the percentage of cells that accumulated in the G_0-G_1 phase. Therefore, at this concentration, the predominant effect of NO-ASA is to induce apoptosis.

The induction of the atypical cells in response to NO-NSAIDs was intriguing. This phenomenon, which is currently under investigation, requires further assessment. It is important to note, however, that traditional NSAIDs, even at high concentrations, failed to induce this morphologically unique cell type. It is reasonable to speculate that cellular forms devoid of apparent DNA, akin to platelets, have a shortened life span, which may contribute in a unique way to the dramatic effect of NO-NSAIDs on cell kinetics.

All three NO-NSAIDs were superior to their parent NSAIDs in affecting the parameters we studied. However, they differed substantially among themselves when compared on an equimolar basis; NO-ASA was clearly the most effective in all respects. Given the structural complexity of these molecules and the paucity of information on the biological role of each of their structural components (traditional NSAID, spacer, $-NO_2$), it is difficult to deduce plausible structure-activity correlates that could account for such pronounced differences. Remarkably, of the three parent NSAIDs, ASA has the highest IC_{50}, followed by ibuprofen and sulindac. However, of the NO derivatives, NO-ASA has by far the lowest IC_{50}.

It is unclear at this point what accounts for the enhanced effectiveness of NO-NSAIDs compared with their NSAID counterparts. The magnitude of their enhanced activity, especially that seen with NO-ASA, is difficult to ascribe to simple changes in the physical properties of each NSAID. The spacer part of the molecule and the $-NO_2$ group, which releases NO, must contribute to this effect. Our preliminary data indicate that these compounds do not block PG synthesis, at least at concentrations that inhibit cell growth. Combined with their effect on HCT-15 cells that lack both COX isozymes (12), this suggests that the effect of NO-NSAIDs does not necessarily involve inhibition of COX, the classical target of traditional NSAIDs and of the selective COX-2 inhibitors. Rather, it appears likely that NO-NSAIDs act on targets beyond COX and may use novel modes of action. Such a notion has been considered for traditional NSAIDs as well (13).

An important biological question is whether NO-NSAIDs maintain their superior performance *in vivo*. Although it is difficult to extrapolate results from cultured cells to animal systems and humans, a recent study suggested that NO-NSAIDs may be more effective *in vivo* than traditional NSAIDs. The chemopreventive effect of aspirin *versus* a NO-ASA derivative (NCX 4016; a positional isomer of the one reported here) was assessed using a rat azoxymethane model of colon cancer (14). Whereas ASA reduced the number of aberrant crypt foci, the putative preneoplastic lesions of the colon, by 64%; NO-ASA reduced them even more (85%). Further work, some of it already in progress, will assess this critical question. Of interest, it was recently reported that low-dose ASA ($<$150 mg) used for cardiac prophylaxis failed to protect patients from colon cancer (15). NO-NSAIDs, with their potential for enhanced effectiveness against colon cancer, may provide double protection against coronary artery disease and colon cancer.

In conclusion, our data demonstrate that a novel class of NSAID derivatives, the NO-NSAIDs, which promise to be less toxic than traditional NSAIDs, affects colon cancer cell kinetics *in vitro* in a way consistent with a chemopreventive effect. In this respect, NO-NSAIDS are severalfold more powerful than traditional NSAIDs. Their potential role in human colon cancer prevention remains to be established.

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