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

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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_{1}/G_{2} 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 (2) 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 (7, 8) would alleviate these problems, 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-cm² 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-(methylsulfonyl)phenyl)methylene]-1H-indene-3-acetic acid 4-(nitrooxy)butyl ester), NO-ibuprofen (NCX2210; trans-3-[4-[4-(methylpropyl)benzenesyneacetyl]x]-3-methoxyphenyl]-2-propenoic acid 4-nitrooxy)butyl ester), and NO-aspirin (NCX4040; 2-(acetoxybenzoic 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.
Work station (Coulter) using the software programs Multigraph and Multi-cycle.

Cell Proliferation and Cell Cycle Analysis. Cells (0.5 × 10^6) were fixed in 100% methanol for 10 min at 2°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 G0-G1, G2-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-G0-G1) 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 IC50 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Aspirin</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>NO-aspirin (NCX 4040)</td>
<td>1</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>NO-ibuprofen (NCX 2210)</td>
<td>42</td>
</tr>
<tr>
<td>Sulindac</td>
<td>750</td>
</tr>
<tr>
<td>NO-sulindac (NCX 1102)</td>
<td>150</td>
</tr>
</tbody>
</table>

* Exceeded the maximum concentration used in these studies.
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...
cells in S and G2-M phases. This effect was concentration depend-
was accompanied by corresponding reductions of the proportion of
cells, inducing a block in the G0-G1 to S transition. This was
NSAIDs significantly altered the cell cycle distribution of HT-29
methods were always concordant.
determined by morphological criteria rather than by cell DNA
ibuprofen, and 1.9-fold for NO-sulindac. As expected (10, 11), the
NO-NSAID for 48 h, the increase in the percentage of apoptotic
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 G0-G1 to S transition. This was
evident by the increased percentage of cells in the G0-G1 phase that
was accompanied by corresponding reductions of the proportion of
cells in S and G2-M phases. This effect was concentration depend-
ent. NO-ASA was the most effective of the three in inducing these
changes. This is evident, for example, when equimolar concentra-
tions of the three compounds are compared, e.g., exposure to 100
μM for 48 h. NO-ASA increased the G0-G1 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 G2-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 accu-
rately 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 G0-G1 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 substan-
tially 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 informa-
tion on the biological role of each of their structural components
(traditional NSAID, spacer, −NO2), 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 IC50, followed by ibuprofen and sulindac. However, of the NO
derivatives, NO-ASA has by far the lowest IC50.

It is unclear at this point what accounts for the enhanced effective-
ness 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 prop-
ties of each NSAID. The spacer part of the molecule and the −NO2
group, which releases NO, must contribute to this effect. Our preli-
nary 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 tradi-
tional 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 pneumo-
plastic 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 effec-
tiveness 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 tradi-
tional NSAIDs, affects colon cancer cell kinetics in vitro in a way con-
sistent 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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