

NO-donating nonsteroidal antiinflammatory drugs (NSAIDs) inhibit colon cancer cell growth more potently than traditional NSAIDs: a general pharmacological property?

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Abstract

The novel nitric oxide-donating nonsteroidal antiinflammatory drugs (NO-NSAIDs), consisting of a traditional NSAID to which a NO releasing moiety is covalently attached, may have an important role in colon cancer prevention and/or treatment. Preclinical studies have shown that NO-aspirin (NO-ASA) is more potent than traditional ASA in preventing colon cancer. Preclinical and clinical studies have also documented its superior safety, compared to traditional ASA. To evaluate the role of this structural modification on the cancer cell growth inhibitory effect of NSAIDs, we studied seven pairs of traditional NSAIDs (ASA, salicylic acid, indomethacin, sulindac, ibuprofen, flurbiprofen, piroxicam) and their corresponding NO-NSAIDs. All NO-NSAIDs (except NO-piroxicam which is a salt and not a true NO-NSAID) have greater potency in inhibiting HT-29 and HCT-15 colon cancer cell growth compared to their NSAID counterparts: the IC₅₀s of the NO-NSAIDs were enhanced between 7- and 689-fold in HT-29 cells and 1.7- to 1083-fold in HCT-15 cells over those of the corresponding NSAIDs. Their growth inhibitory effect is due to a profound cell kinetic effect consisting of reduced cell proliferation and enhanced cell death. Since HT-29 cells express cyclooxygenases but HCT-15 do not, this effect appears independent of cyclooxygenase in the colon cancer cells. Thus the structural modification of these traditional NSAIDs leading to NO-NSAIDs enhances their potency in inhibiting colon cancer cell growth. Our findings suggest that the enhanced potency imparted on NSAIDs by this structural modification represents a pharmacological property that may be a general one for this class of compounds.

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1. Introduction

NO-donating nonsteroidal antiinflammatory drugs (NO-NSAIDs) are a highly promising novel class of drugs that may impact several areas of modern pharmacology and therapeutics. Data from several laboratories indicate that NO-NSAIDs could be effective in a variety of diseases

including cardiovascular, rheumatological and lung diseases, Alzheimer's disease, and cancer [1,2]. These compounds appear to be much safer compared to their parent compounds [3].

We have recently reported that, compared to their traditional counterparts [4], three NO-NSAIDs (NO-aspirin, NO-sulindac, NO-ibuprofen) inhibit the growth of cultured human colon cancer cells more potently than traditional NSAIDs [5]. Since chronic use of traditional NSAIDs reduces the incidence of, and mortality from colon cancer by about half [6], one might expect that NO-NSAIDs could be at least as effective. Indeed, NO-ASA was very effective in inhibiting intestinal carcinogenesis in *Min* mice [7] and

Abbreviations: NSAIDs, nonsteroidal antiinflammatory drugs; ASA, aspirin; NO-NSAIDs, NO-donating NSAIDs

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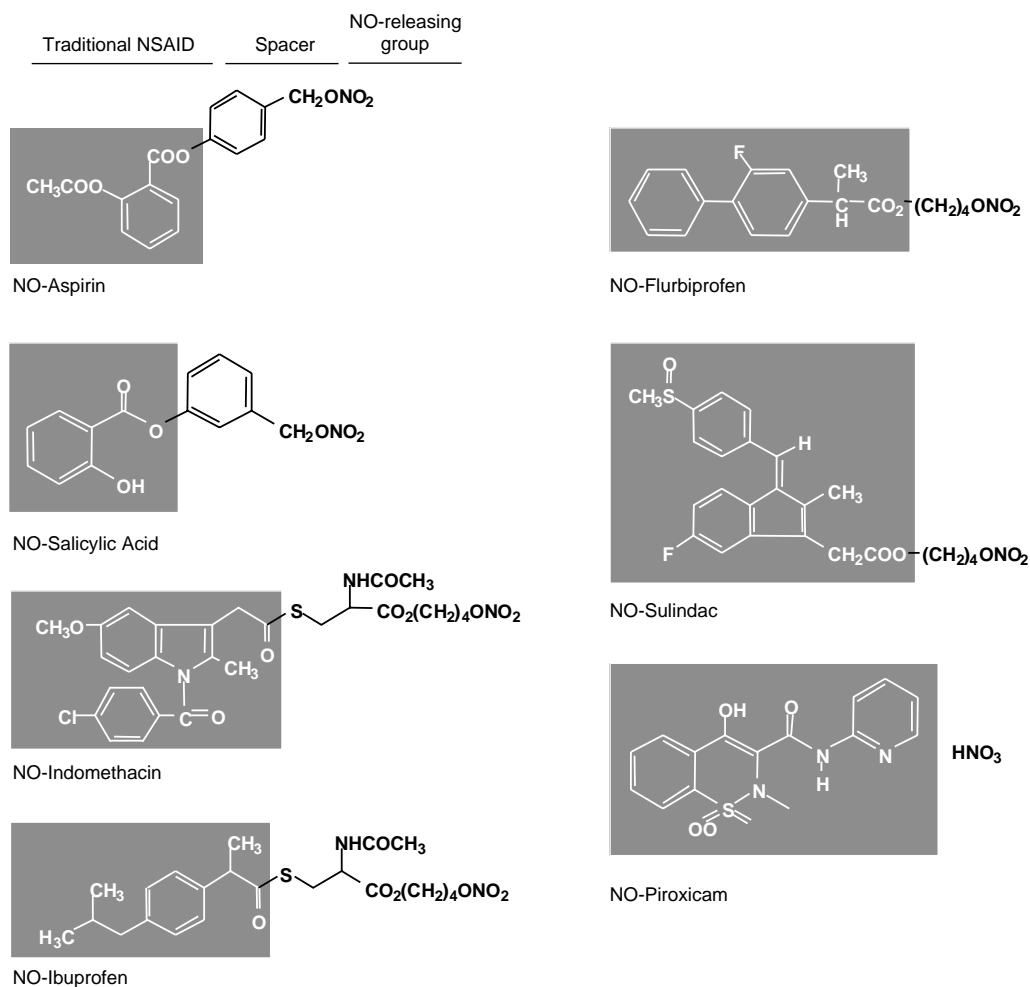


Fig. 1. The chemical structures of NO-NSAIDs. The three structural components of NO-NSAIDs are indicated: the traditional NSAID is shown in the shaded box, the spacer molecule links the traditional NSAID to $-\text{NO}_2$, which can release NO.

when studied in a rat model of colon cancer, NO-ASA was more effective than traditional ASA in preventing colon carcinogenesis [8]. Thus, study of the effect of NO-NSAIDs on colon cancer cells becomes potentially significant.

Traditional NSAIDs are a large and diverse family of pharmacologically useful compounds, classified into several subgroups based on their chemical structure [9]. Therefore, it would be mechanistically important to know whether the enhanced activity observed in the three NO-NSAIDs already reported on, is a generalized property of NSAIDs or it is simply restricted to only these three compounds. Interestingly, we observed that the enhanced activity of the three NO-NSAIDs that we studied is not uniform. In fact, this enhancement in activity ranges between 30- and >5000-fold, and no apparent structural explanation exists for this wide variation. Therefore, study of NO-NSAIDs representing other classes of NSAIDs might provide insights into the principles underlying this variability.

Based on these considerations we studied the effect of several NO-NSAIDs on the growth of human colon cancer cells. The NSAIDs from which they were derived belong to

the following structural classes: *salicylic acid derivatives*: ASA and salicylic acid; *indole and indene acetic acids*: indomethacin and sulindac; *arylpropionic acids*: ibuprofen and flurbiprofen; and *enolic acids*: piroxicam. The chemical structures of these are shown in Fig. 1. We demonstrate that all NO-NSAIDs have greater efficacy and potency in inhibiting colon cancer cell growth compared to their traditional NSAID counterparts; that their growth inhibitory effect is due to a profound cell kinetic effect; and that their effect appears independent of cyclooxygenase. These data, taken together with work on other cancer cell lines [10], indicate that the enhanced efficacy brought about by this structural modification of traditional NSAIDs likely represents a general property.

2. Methods

2.1. Reagents

NO-aspirin (NCX4040): 2-(acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester; NO-ibuprofen (NCX2210):

trans-3-{4-[α -methyl-4-(2-methylpropyl)benzeneacetyl]phenyl}-2-(propenoic acid 4-nitrooxy)-butyl ester; NO-flurbiprofen (*HCT 1026*): 2-Fluoro- α -methyl[1,1'-biphenyl]-4-acetic acid 4-(nitrooxy)butyl ester; NO-indomethacin (*NCX2121*): (*S*)-*N*-acetyl-[-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-acetyl]-cysteine 4-(nitrooxybutyl) ester; NO-piroxicam (*NCX1301*): 4-Hydroxy-2-methyl-*N*-2-pyridinyl-2*H*-1,2-benzothiazine-3-carboxamide 1,2-dioxide nitrate; NO-salicylic acid (*NCX4023*): 2-Hydrobenzoic acid 3-(nitrooxymethyl)phenyl ester; and NO-sulindac (*NCX1102*): (*Z*)-5-Fluoro-2-methyl-1-{[4-(methylsulfinyl)phenyl] methylene}-1*H*-indene-3-acetic acid 4-(nitrooxy)butyl ester were synthesized by NicOx, SA, France. The corresponding NSAIDs were from Sigma Chemical Co. (St. Louis, MO). Stock (100 mM) solutions of NO-NSAIDs and NSAIDs were prepared in DMSO (Fisher Scientific, Fair Lawn, NJ). Final DMSO concentration was adjusted in all media to 1%.

2.2. Cell lines

All cell lines were from American Type Tissue Collection, Rockville, MD and their recommendations were followed. HT-29 and HCT-15 human colon adenocarcinoma and HUV-EC-C human endothelial cell lines were grown and treated as previously described [5]. Cells were counted using a hemacytometer. Viability was determined by the trypan blue dye exclusion method.

2.3. Cell proliferation

Cells (0.5×10^6) were fixed in 100% methanol for 10 min at -20°C , pelleted (5000 rpm \times 10 min at 4°C), resuspended and incubated in PBS containing 1% FBS/0.5% NP-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 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-anti-mouse-phycoerythrin antibody (diluted 1:50) for 60 min at room temperature in the dark. Flow 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 Work station (Coulter) using the Software programs MultigraphTM and MulticycleTM.

2.4. Phase contrast microscopy

Phase contrast images were captured on an Olympus IX50 microscope fitted with a Hitachi KP-D50 color digital camera and processed using Flashpoint 3D software 2.0

(Integral Technologies). Cells were imaged after 48 h of treatment and just prior to cell counting.

2.5. Transmission electron microscopy

Control HT-29 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 $10,000 \times g$ 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.

2.6. Assay for apoptosis

The induction of apoptosis was determined 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. Atypical cells maintain their basic cellular configuration but show progressive loss of nuclear material, which in extreme cases is totally lost [5].

2.7. Statistical analyses

Data are presented as means \pm S.E.M. for different sets of plates and treatment groups, as indicated. Statistical comparison among the groups was performed using a one-way ANOVA followed by the least significant difference method.

3. Results

3.1. Effect of NO-NSAIDs and NSAIDs on colon cancer cell growth

HT-29 colon cancer cells, seeded in 6-well plates at a density of 2.5×10^4 cells/cm², were exposed to various concentrations of NSAIDs or NO-NSAIDs for 48 h. IC₅₀ values were calculated from the growth curves. Table 1 and Fig. 2 summarize our findings.

With the possible exception of piroxicam, all NO-NSAIDs inhibited the growth of colon cancer cells more potently than their corresponding NSAIDs. The IC₅₀s of

Table 1
IC₅₀ values of NSAIDs and NO-NSAIDs in colon cancer cells

Compound	IC ₅₀ (μM)	
	HT-29	HCT-15
Aspirin	3500 ± 300	3000 ± 250
NO-aspirin	5 ± 2*	3 ± 1
Ratio	689 ± 115	1083 ± 114
Sulindac	682 ± 35	487 ± 50
NO-sulindac	33 ± 5*	35 ± 4*
Ratio	21 ± 1	14 ± 0.7
Indomethacin	580 ± 50	436 ± 40
NO-indomethacin	35 ± 4*	25 ± 3*
Ratio	17 ± 0.7	18 ± 0.8
Salicylic acid	>1000 [†]	>1000 [†]
NO-salicylic acid	143 ± 28	112 ± 18
Ratio	>7	>9
Ibuprofen	>1000 [†]	>1000 [†]
NO-ibuprofen	48 ± 15	57 ± 20
Ratio	>21	>18
Piroxicam	>1000 [†]	>1000 [†]
NO-piroxicam	>1000 [†]	842 ± 65
Ratio	>1	>1.2
Flurbiprofen	782 ± 35	450 ± 50
NO-flurbiprofen	98 ± 10*	285 ± 75
Ratio	9 ± 0.4	1.7 ± 0.2

Cells were treated with various concentrations of NO-(aspirin, sulindac, salicylic acid, flurbiprofen, ibuprofen, piroxicam, and indomethacin) and their corresponding traditional NSAIDs as described in Section 2. Cell numbers were determined at 48 h from which IC₅₀ values were calculated. Results are mean ± S.E.M. of three to five different experiments done in duplicate. [†]Exceeded the maximum concentrations used in these studies.

* $P < 0.001$ compared to the corresponding traditional NSAID.

the NO-NSAIDs were enhanced between 7- and 689-fold in HT-29 cells and 1.7- to 1083-fold in HCT-15 cells over those of the corresponding NSAIDs. The greatest enhancement of biological activity was observed for ASA (1083-fold) and the lowest for flurbiprofen (1.7-fold). For several compounds the IC₅₀ could not be determined with accuracy, as their growth curves did not reach 50% growth inhibition. Consequently, these values are presented as “greater than” the highest concentration used; this is reflected in the corresponding IC₅₀ ratios between the traditional NSAID and its NO-NSAID counterpart. Nevertheless, the growth curves of piroxicam and NO-piroxicam appear similar (data not shown) and it is likely that they behave similarly in terms of their effect on HT-29 cell growth.

Viewed in terms of chemical classes, these results show that this structural modification had its strongest effect on ASA, whereas its weakest effect was on piroxicam. NO-piroxicam, however, is only a salt of traditional piroxicam and not a true NO-NSAID (Fig. 1). For all other com-

pounds, the effect of this structural modification was roughly similar, bringing about a 10- to 20-fold increase in potency with respect to cell growth inhibition.

The inhibitory effect of the NO-NSAIDs on cell growth was accompanied by profound morphological changes. Compared to control cells, NO-NSAIDs had a pronounced effect on cell density and cell morphology as assessed by light microscopy. Cells were shrunken, rounded and with compromised anchorage to the culture plate. For example, as shown in Fig. 3, although indomethacin changed the morphology of HT-29 cells, the changes induced by NO-indomethacin involved the overwhelming majority of cells (all the cells in that field) and were much more pronounced. Similar changes, but varying in degree, were observed with all other NO-NSAIDs. HCT-15 cells showed morphological changes similar to those of HT-29 cells (data not shown).

3.2. NO-NSAIDs alter colon cancer cell kinetics

In order to evaluate the mechanism involved in the reduction of cell growth, we analyzed the effect of each of these compounds on cell renewal and cell death, two determinants of cell growth. We evaluated cell proliferation by measuring PCNA expression and cell death by morphological assessment of DAPI-stained cells following exposure to these compounds.

Treatment of HT-29 cells with NO-NSAIDs was accompanied by a significant antiproliferative effect and by induction of apoptosis (Fig. 4). In all cases, both the decrease in PCNA expression and the induction of apoptosis were concentration-dependent. The most pronounced changes were noted with NO-ASA followed by the two arylpropionic acid derivatives, NO-ibuprofen and NO-flurbiprofen. In this respect, NO-ASA was by at least an order of magnitude more potent than any of the remaining NO-NSAIDs.

Unlike the other NO-NSAIDs, NO-ASA induced three morphologically distinct populations of cells on DAPI stained samples: cells without morphological changes (“unchanged”), apoptotic cells, and atypical cells, as defined in Section 2 and shown in Fig. 3; we have described these cells previously [5,10]. To assess whether the induction of the atypical cell by NO-ASA is cell type-restricted, we evaluated the effect of NO-ASA and ASA on HUV-EC-C cells, which are derived from normal human vascular endothelium and are not tumorigenic in immunosuppressed mice [11]. Fig. 5 demonstrates that NO-ASA induces atypical cells in this cell line as well, suggesting that this property of NO-ASA is independent of target cell type.

3.3. NO-NSAIDs induce morphological changes in HT-29 cancer cells

Both the light microscopic findings and DAPI-stained cells make it clear that treatment of HT-29 cells with

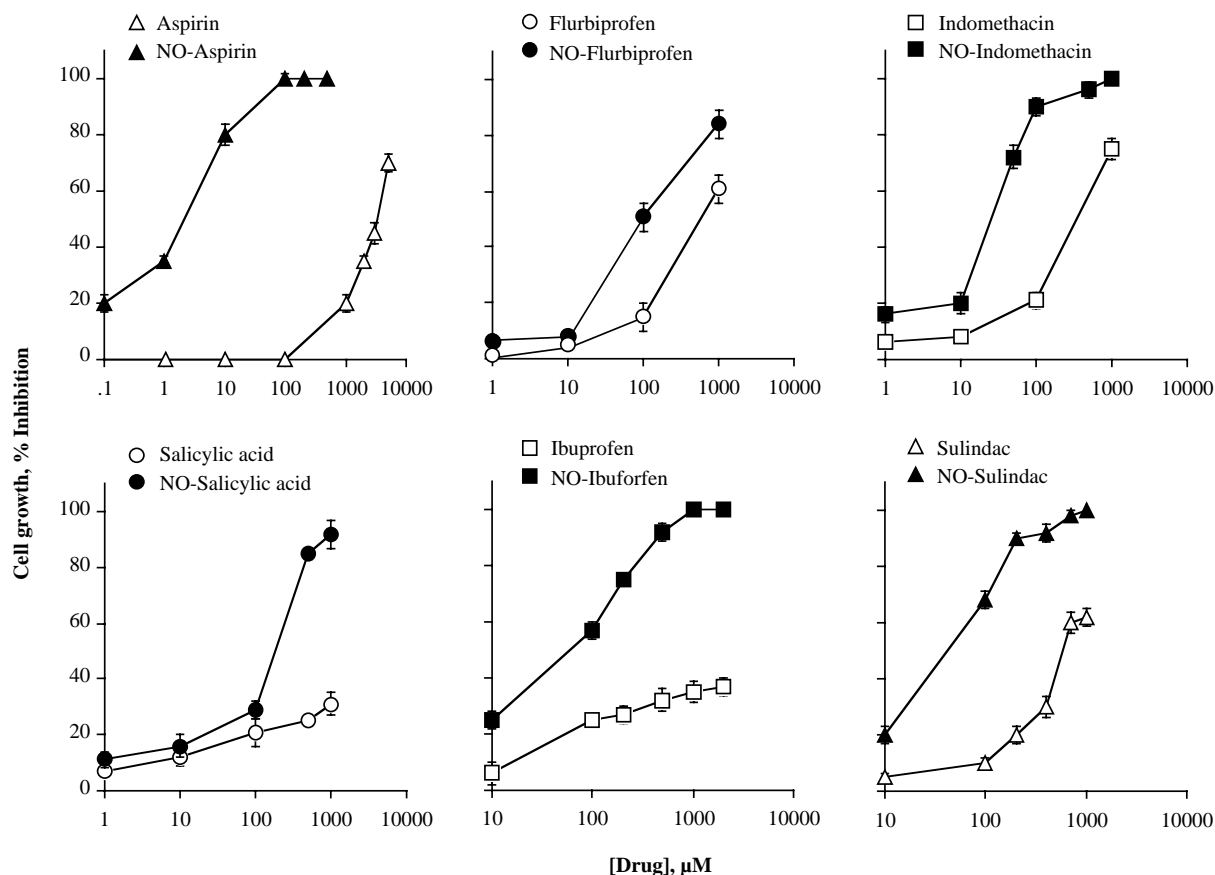


Fig. 2. Effect of NO-NSAIDs on HT-29 cell growth. Cells were treated with various concentrations of NO-NSAIDs and their corresponding traditional NSAIDs for 48 h as described in Section 2. Results represent mean \pm S.E.M. of at least three different experiments with duplicate plates. See Table 1 for IC_{50} values \pm S.E.M.

NO-NSAIDs leads to significant morphological changes. We 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 colon cancer cells (Fig. 3). ASA at 5 mM induces features of apoptosis with nuclear condensation, as previously reported [12]. NO-ASA, 100 μ M, causes extensive vacuolization of the cytoplasm and loss of the integrity of the 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. These changes are strongly suggestive of cell necrosis.

3.4. 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 [5,13]. The two colon cancer cell lines that we studied, HT-29 and HCT-15, differ in their expression of COX; the former expresses both COX-1 and -2, which are catalytically active, whereas the latter does not [14]. The results shown in Table 1 indicate that, with the exception of NO-flurbiprofen, the IC_{50} s of each NO-NSAID

are similar for both cell lines. In fact, the HCT-15/HT-29 ratios of the IC_{50} s range between 0.86 (NO-ibuprofen) and 1.57 (NO-ASA), except for NO-flurbiprofen, for which this ratio is 0.19. These findings indicate that the presence of COX is not required for the growth inhibitory effect of these compounds on colon cancer cells.

4. Discussion

A significant body of work indicates that NO-donating compounds often have enhanced pharmacological activity compared to their parent compounds [2]. We undertook a systematic study of the effect of this structural modification on NSAIDs, focusing on representative members of this large and clinically important family of compounds. Our results (a) demonstrate that this structural modification imparts enhanced potency on all traditional NSAIDs studied, and (b) strongly suggest that this is a general pharmacological property of NO-NSAIDs.

Given the efficacy of NSAIDs as chemopreventive agents against colon cancer, which was recently formally documented for ASA by two human interventional trials [15,16], we examined the effect of this structural modification of NSAIDs on cell growth. Cell growth represents a

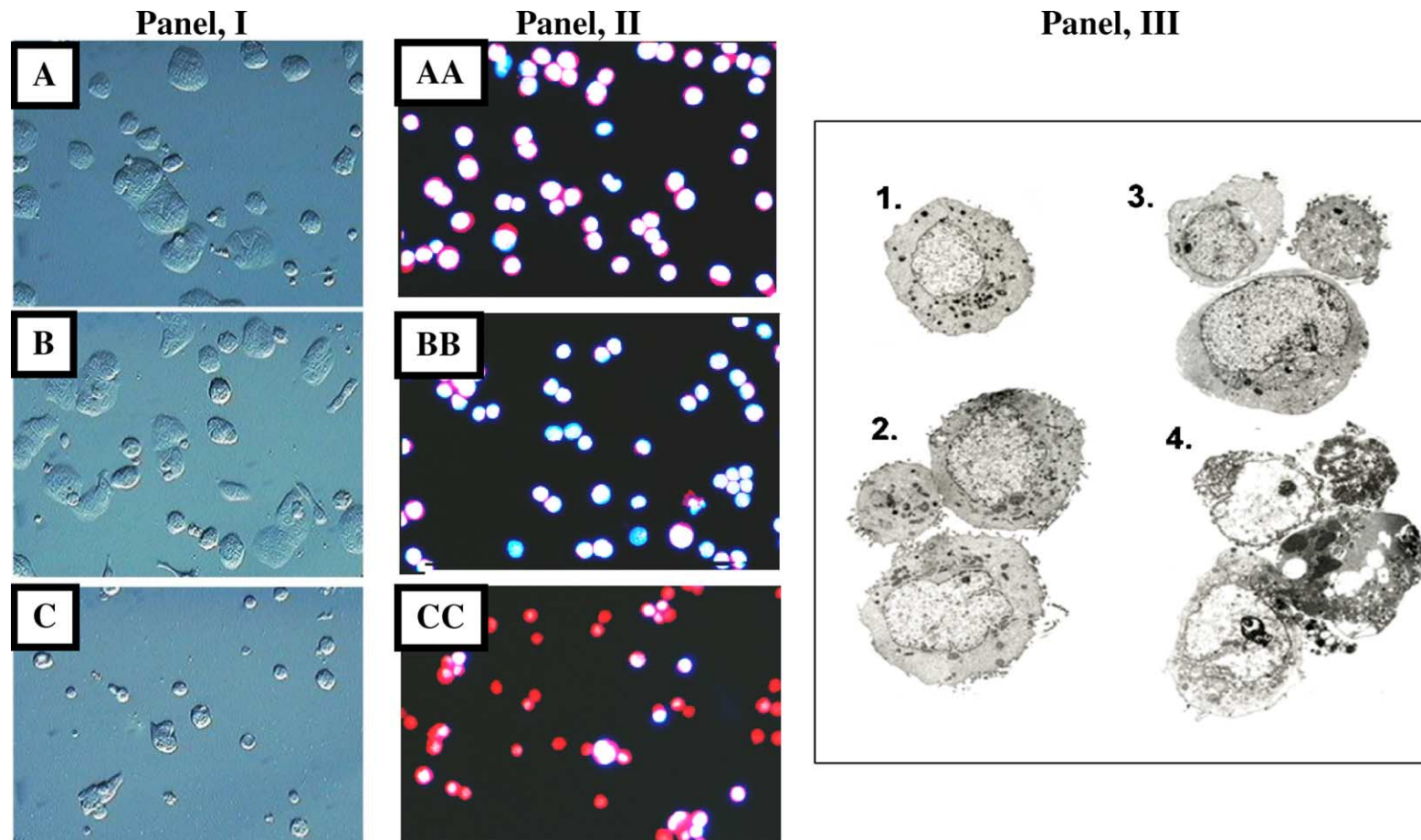


Fig. 3. Effect of NO-NSAIDs on morphology and apoptosis in HT-29 cells. All were exposed for 48 h and evaluated as in Section 2. (Panel I) Light microscopy: (A) control cells (no drug); (B) treated with indomethacin 1000 μM ; (C) treated with NO-indomethacin 100 μM . (Panel II) DAPI-stained cells: (AA) control cells (untreated); (BB) cells treated with ASA 5000 μM for 24 h; (CC) cells treated with NO-ASA 1 μM . (Panel III) Electron micrographs: (1) control (no drugs); (2) cells treated with ASA 5000 μM ; (3) cells treated with NO-ASA 1 μM ; (4) cells treated with NO-ASA 10 μM . Magnification, 1000 \times .

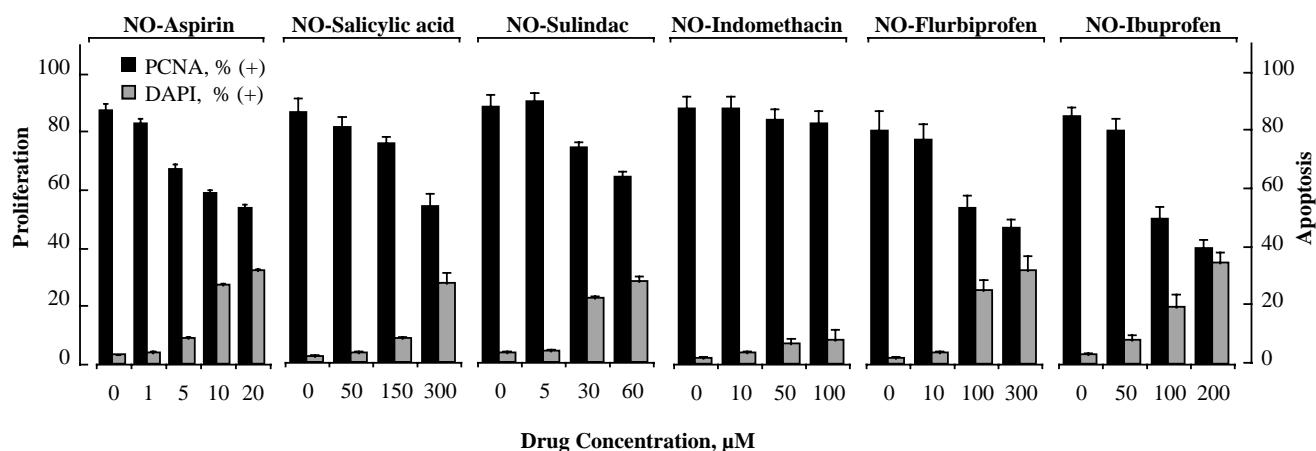


Fig. 4. The effect of NO-NSAIDs on HT-29 cell proliferation and apoptosis. Cells were treated with the respective NO-NSAIDs for 48 h. PCNA expression and apoptosis were determined as described in Section 2. Results are mean \pm range of two different experiments done in duplicate.

most critical parameter for the development of cancer, as it determines whether a “tumor”, i.e. a positive balance of cells, will develop in the colon. All NO-NSAIDs showed enhanced potency with respect to this effect. Of the seven NO-NSAIDs that we evaluated, NO-piroxicam is the only one that showed marginally enhanced potency compared to traditional piroxicam. As already discussed, NO-piroxicam does not represent a “true NO-NSAID” in that it lacks the covalent linkage of the NO-releasing moiety to the NSAID molecule. Thus, in our analysis of these data below, when we refer to NO-NSAIDs as a group, NO-piroxicam will not be included.

Three lines of evidence suggest that the enhanced potency of NSAIDs following their structural modification is a general property. First, the six NO-NSAIDs that display this property belong to three different chemical groups: salicylic acid derivatives; indole and indene acetic acids; and arylpropionic acids. They are structurally diverse, yet their potency with respect to cell growth is consistently enhanced, roughly 10-fold or more. Second, NO-piroxicam, an enolic acid derivative with the structural difference mentioned above, showed no enhanced potency. This compound can be viewed as a negative control that underscores the validity of the argument. And, finally,

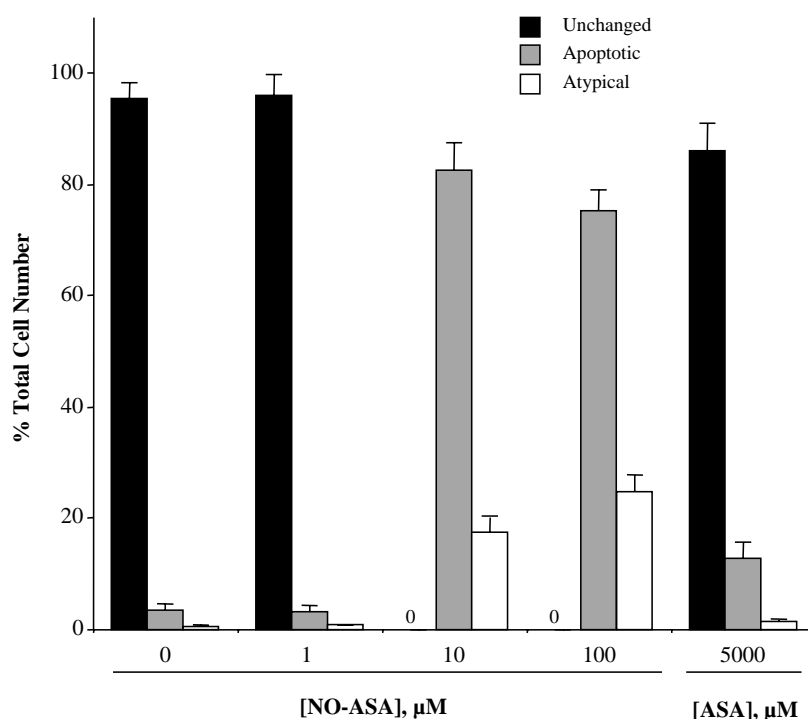


Fig. 5. The effect of NO-ASA on HUV-EC-C vascular endothelial cells. Cells were treated with various concentrations of ASA and NO-ASA for 72 h and stained with DAPI as described in Section 2. Results are mean \pm S.E.M. of three different experiments done in duplicate.

published experience with other NO-donating compounds is consistent with this concept. For example, NO-steroids and NO-paracetamol are more potent than their parent compounds [17,18].

Although there are individual variations, the overall mechanism of action of NO-NSAIDs appears to share common elements among them, at least as this can be assessed by the parameters that we monitored. First, all have a significant effect on cell kinetics inhibiting cell proliferation and inducing cell death by apoptosis. Of note, all parent NSAIDs inhibited the growth of both colon cancer cell lines and are known to affect their kinetic properties [4,19]. And, second, the effect of all compounds tested on cancer cell growth appears to be independent of COX-1 and -2. All compounds had similar IC_{50} s for the two cell lines, HT-29 and HCT-15, which differ in their expression of COX. NO-flurbiprofen is a possible exception; in all other NO-NSAIDs the ratio of HCT-15 IC_{50} over HT-29 IC_{50} ranges between 0.7 and 1.6, whereas for NO-flurbiprofen it is 0.2. It is unclear what accounts for the variation in the cell growth inhibitory potency of these compounds. As can be seen in Fig. 1, there is no real pattern correlating the IC_{50} s with any of the structural elements of an NO-NSAID molecule. A detailed structure–activity relationship study, using analogs of these compounds, will be required to address this issue.

The morphological changes induced by these compounds in both cell lines that we studied represent another shared feature of their activity. However, as we were unable to evaluate this effect in normal human colonocytes in vitro (no such line is available), it is not possible to ascertain whether such an effect is restricted to malignant cells or it involves phenotypically normal cells as well.

An interesting observation was the great enhancement of potency that this structural modification imparts on ASA. Remarkably, ASA is the least potent of all seven NSAIDs in inhibiting cell growth. The induction of the atypical cell by NO-ASA appears to be an important property of this molecule that is not restricted to a specific cell type. Our observation on colon cancer cells, combined with similar observations on pancreatic and other cancer cells [10] and, importantly, on the HUV-EC-C cells that are derived from the normal vascular endothelium, argues for a distinguishing pharmacological property. It is likely that the induction of the atypical cell, whatever its mechanism, is an important kinetic effect of NO-ASA that can account for its profound growth inhibitory effect. Overall, the induction of cell death appears to be a more prominent effect of NO-NSAIDs on these cell lines than inhibition of proliferation and may represent a common property targeted by these NO-NSAIDs. It is, therefore, 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. Ongoing work is attempting to address these complex issues. For example, a denitrated analog of NO-ASA that we have synthesized failed to show the

enhanced potency of the full molecule, indicating that the NO-releasing moiety ($-NO_2$) is crucial for its cell growth inhibitory activity in these cell lines ([20] and unpublished observations).

Finally, these data confirm and expand our original observation that, similar to NSAIDs, NO-NSAIDs inhibit cancer cell growth by mechanisms independent of (or in addition to) their COX inhibitory effect [10,14]. The NSAIDs that we studied here in their traditional form inhibit COX in varied ways. For example, ASA covalently modifies COX thus resulting in irreversible inhibition of its activity (suicide inhibitor), whereas most of the others are reversible, competitive inhibitors of COX [9]. Nevertheless, with the possible exception of NO-flurbiprofen, all inhibited cell growth similarly in COX expressing and COX-null cells.

In conclusion, our data strongly suggest that this structural modification of NSAIDs enhances their colon cancer growth inhibitory properties. That this effect is manifest in cancer cell lines of varied tissue origin indicates that these compounds merit further study as a novel class of potentially important chemopreventive and perhaps chemotherapeutic agents.

Acknowledgments

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