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Original article

Therapeutic potential of sulindac hydroxamic acid against human pancreatic and colonic cancer cells

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ABSTRACT

The non-steroidal anti-inflammatory drug (NSAID) sulindac exhibits cyclooxygenase (COX)-dependent and COX-independent chemopreventive properties in human cancer. The present study was aimed at investigating whether the hydroxamic acid substitution for the carboxylic acid group could enhance the in vitro antitumor and antiangiogenic activities of sulindac. Characterization tools used on this study included analyses of cell viability, caspase 3/7 induction, DNA fragmentation, and gene expression. Our findings demonstrate that the newly synthesized hydroxamic acid derivative of sulindac and its sulfone and sulfide metabolites were characterized by a good anticancer activity on human pancreatic and colon cancer cells, both in terms of potency (IC_{50} mean values from 6 ± 1.1 μM to 64 ± 1.1 μM) and efficacy (E_{max} of ~100%). Hydroxamic acid derivatives trigger a higher degree of apoptosis than carboxylic acid counterparts, increase bax/bcl-2 expression ratio and induce caspase 3/7 activation. Most notably, these compounds significantly inhibit proangiogenic growth factor-stimulated proliferation of vascular endothelial cell (HUVEC) at sub-micromolar concentrations. Our data also provide evidence that the COX-active metabolite of sulindac hydroxamic acid were the most active of the series and selective inhibition of COX-1 but not COX-2 can mimic its effects, suggesting that COX inhibition could only play a partial role in the mechanism of compound action. In conclusion, these data demonstrate that substitution of the carboxylic acid group with the hydroxamic acid moiety enhances in vitro anti-proliferative, proapoptotic and antiangiogenic properties of sulindac, therefore increasing the therapeutic potential of this drug.

1. Introduction

Inflammatory processes have been shown to enhance carcinogenesis and tumor progression in multiple ways including production of reactive oxygen species and induction of vascular endothelial growth factor. Following the initiation of solid tumors, the cross-talk between transformed epithelial and stromal cells has a key role in promoting cancer progression. For example, in stromal cells, proinflammatory cytokines induce up-regulation of COX-2 which, in turn, may contribute to cancer development by producing adhesion molecules and activating neoangiogenesis, particularly through the production of prostanoids [1]. These notions highlight that effective cancer chemopreventive and/or therapeutic agents should be able to interfere with different elements which play a pivotal role into the tumor–host interface circuit.

Sulindac is a non-steroidal anti-inflammatory drug (NSAID) of the arylalkanoic acid class that, in vivo, is irreversibly oxidized to sulindac sulfone or reversibly converted into its anti-inflammatory active compound, sulindac sulfide. Sulindac and sulindac sulfone are devoid of any activity as COX inhibitors, while most of the biological effects of the sulfide metabolite are dependent upon inhibition of both cyclooxygenase-1 (COX-1) and COX-2 activities and reduction in prostaglandin (PG) synthesis [2]. Compelling evidence has been provided on the therapeutic potential of sulindac as chemopreventive agent in colorectal cancer and both COX-dependent and COX-independent mechanisms appear to contribute to such an effect [3]. In particular, it has been recently demonstrated that induction of cytochrome P450 and carcinogen detoxification enzymes such as NAD(P)H:quinine oxidoreductase and glutathione S-transferase may account for the chemopreventive action of sulindac and its metabolites in human colon

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cancer cells [4]. The antitumor properties of sulindac were mostly due to the ability of sulfone and sulfide metabolites to negatively interfere with Ras signaling in tumor cells [5–7] and with angiogenetic pathways in the surrounding tissue [8]. Finally, several lines of evidence also indicate that sulindac is able to potentiate the effect of different chemotherapeutic agents including cisplatin, cyclophosphamide, melphalan, carmustine and doxorubicin [9]. Studies have shown that most, if not all, NSAIDs cause increased risk of heart attack in humans and such an effect can be related to the inhibition of the inducible COX-2 isoform in the cardiac tissue [10]. Noteworthy, sulindac confers ischemic protection, in vitro and in vivo, through late preconditioning mechanisms which did not involve its role as a COX inhibitor [11].

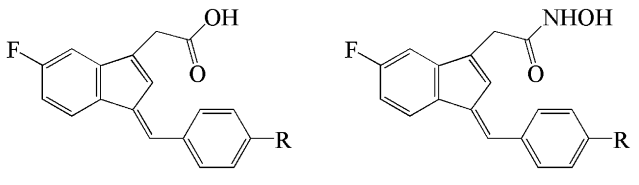
It has been suggested that incorporating a chelating group (hydroxamic acid) into the structure of conventional COX inhibitors resulted in the acquisition of antioxidant properties which might increase the therapeutic benefit of these drugs because of implication of free radical production in the pathogenesis of cancer and ischemic-reperfusion injury [12]. Furthermore, recent findings demonstrate that sulindac and some hydroxamic acids synergistically induce apoptosis in human cancer cells [13], suggesting that the chemical modification of sulindac structure might be a reasonable strategy for the development of new compounds with enhanced antitumor activity. Overall, these data prompted us to synthesize and characterized the in vitro antitumor, proapoptotic and antiangiogenic properties of sulindac hydroxamic acid and its sulfone and sulfide metabolites, in order to take into account all chemical species potentially involved in the mechanism of drug action, in vivo. Sulindac, sulindac sulfone and sulindac sulfide were evaluated for comparison (Fig. 1).

2. Results

2.1. Chemistry

The acids **b**,**c** were prepared starting from sulindac (**a**) [14] by using the following steps (Scheme 1) according to the literature for the same compounds [15]. The reaction of **a** (commercially available), with the oxone, in a mixture of THF/MeOH 1:1 at room temperature afforded sulindac sulfone (**b**). The reaction of **a** in the presence of Ph_3P and $TiCl_4$ (solution 1 M in CH_2Cl_2) in THF at room temperature afforded sulindac sulfide (**c**).

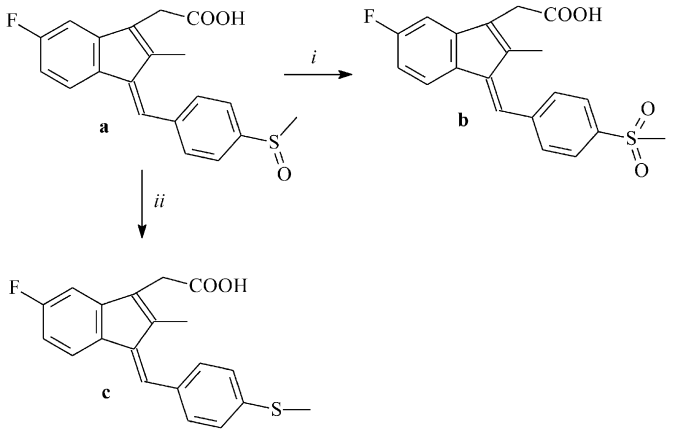
The hydroxamic acids **1**–**3** were prepared starting from **a** [14] by using the following steps (Scheme 2) according to the literature for compounds with similar structure [15]. The reaction of **a** with $TBDMSiONH_2$ (O-(tert-butyl-dimethylslilyl)hydroxilamine), in the presence of EDCI (N-3,dimethylaminopropyl-N-ethylcarbodimmide hydrochloride), gave the O-silylated hydroxamates **4**–**6**, which were transformed by acid cleavage to the corresponding hydroxamic acids **1**–**3** [16].



a, R= $SOCH_3$
b, R= SO_2CH_3
c, R= SCH_3

1, R= $SOCH_3$
2, R= SO_2CH_3
3, R= SCH_3

Fig. 1. Chemical structures of sulindac (a), sulfone (b) and sulfide (c) metabolites and their hydroxamic acid derivatives (**1**–**3**).



Scheme 1. Synthesis of sulfone and sulfide metabolites of sulindac. Reagents and conditions: (i) oxone, THF/MeOH 1/1 v/v, 0–25 °C, 24 h; (*ii*) Ph_3P, $TiCl_4$, THF, rt, 24 h.

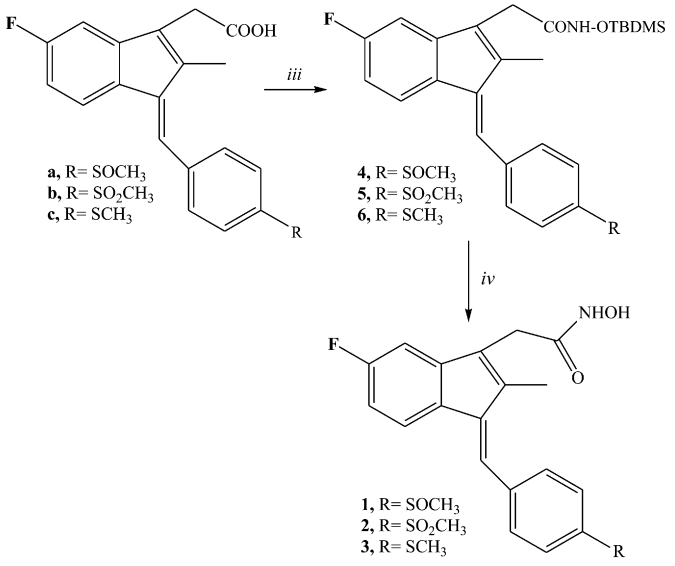
2.2. Pharmacology

2.2.1. Cytotoxicity of compounds against human cancer cells

The antitumour effect in vitro of test compounds was assessed against human cancer cell lines derived from colon (COLO320) and pancreas (MIA PaCa-2). Overall, COLO320 cells were most responsive and exposure of the cells to compounds caused a concentration-dependent decrease in cell viability with mean 50% inhibition (IC_{50}) in the micromolar range of concentrations (Table 1). Within carboxylic acid compounds (**a**–**c**), only sulindac sulfide (**c**) exhibited some anticancer activity, particularly against COLO320 cells (Fig. 2A and C; Table 1). The hydroxamic acid derivatives (**1**–**3**) were more active than their carboxylic acid counterparts, in terms of potency (Table 1), and proven to have a 100% efficacy in both cell lines tested (Fig. 2B and D).

2.2.2. Mechanisms of cell death induced by compounds

Since cytotoxicity assay did not indicate a specific cellular death mechanism, we investigated the ability of selected compounds to induce internucleosomal degradation of genomic DNA (a hallmark



Scheme 2. Synthesis of hydroxamic acid derivatives of sulindac. Reagents and conditions: (iii) $TBDMSONH_2$, EDCI, dry CH_2Cl_2, 0–25 °C, 24 h; (*iv*) TFA, dry CH_2Cl_2, 0 °C, 5 h.

Table 1
IC_{50s} (μM, mean ± SEM; $n = 3$) of test compounds on human cancer cell lines.

Compounds	Cell line	
	MIA PaCa-2	COLO320
a	>200	>200
b	>200	>200
c	146 ± 3.6	38.7 ± 1.2[a]
1	64.1 ± 1.1	62.5 ± 1.2
2	44.4 ± 1.1	26.2 ± 1.2[a]
3	32 ± 1.3	6.0 ± 1.1[a]

[a] Changes in the mean IC50 values obtained for each cell line that are significant ($p < 0.001$; Student's *t*-test).

of apoptosis). While the amount of apoptotic DNA generated upon exposure of both cancer cell lines to sulindac and its metabolites at 200 μM (i.e., the maximum concentration tested in cell viability assay) did not differ significantly from that of untreated controls, hydroxamic acid derivatives were able to induce apoptosis (Fig. 3A and B). In particular, DNA degradation and formation of oligonucleosomal fragments in the cytoplasmic fraction of colonic cells was greater than those observed in pancreatic cancer cells (up to 6-*versus* 15-fold over control, respectively). Noteworthy, **2** caused cell death mainly by apoptosis in both cell lines (apoptosis to necrosis ratio of ~4:1), whereas after treatment with **3**, a switch in the cell death mode from apoptosis to necrosis was observed in MIA PaCa-2 and COLO320 cells (ratio of ~1:1 and 1:12, respectively) (Fig. 3A and B) showing that the extent and the type of cell death were dependent upon the cell line and compound tested.

The possible induction of caspase 3/7 (two enzymes involved in the effector phase of apoptosis) by compounds was also investigated. Treatment of MIA PACa-2 cells with hydroxamic acid derivatives was associated with a significantly increase in the extent of caspase 3/7 stimulation up to 5-fold over control. In COLO320 cells, caspase activation was consistently observed only after incubation with **3**, while the effect of other compounds was negligible (Fig. 3C).

In the same experimental conditions, we evaluated possible alterations in the expression of genes that code for two important proteins involved in apoptosis regulation, Bax (proapoptotic) and Bcl-2 (antiapoptotic), after treatment of cancer cells with **c** and its hydroxamic acid derivative, **3** (i.e., the most active compounds tested). Although compounds **c** and **3** induced different modulation of bax and bcl-2 gene expression in MIA PACa-2 cells, the bax:bcl-2 (proapoptotic:antiapoptotic) expression ratio after treatments was similar to each other and to that of controls (Fig. 4A). Noteworthy, such an effect was in line with the level of caspase 3/7 induction observed in MIA PaCa-2 cells after treatment with these two compounds. In COLO320 cells, bax:bcl-2 expression ratio was almost superimposable in **c** and control samples, while it increased by 130% after treatment with **3** (Fig. 4B). These findings fit well with the present data showing the more pronounced ability of **3** than **c** to promote caspase 3/7 induction in COLO320 cell line.

2.2.3. Involvement of COX enzymes in the effect induced by hydroxamic acid derivatives

Expression of constitutive COX-1 and inducible COX-2 isoforms was confirmed by RT-PCR. As expected, relatively low COX-1 mRNA levels was detected in both cell lines, while COX-2 was expressed only in the human colonic cells (Fig. 5A and B). Experiments with selective COX inhibitors on human cancer cells were performed to investigate the possible contribute of these enzymes to the mechanism of compound action. The concentration–response curves showed that SC560 (COX-1) was active with a potency similar to that of **3**, particularly in COLO320 cells, while no effect was observed after treatment with DFU (COX-2) up to 200 μM (Fig. 5C and D; Table 2). These data suggest that the inhibition COX-1 but not COX-2 might play a partial role in the mechanism of compound action.

To support such a hypothesis, we also considered the possibility that cytotoxic effect induced by compounds can be due to the increased levels of the prostaglandin precursor arachidonic acid (AA) resulting from inhibition of COX, as previously reported [17]. We found that AA was cytotoxic against both pancreatic and colonic cell lines (Fig. 6A). In particular, AA at 200 μM for 48 h caused cell death with an apoptosis to necrosis ratio of 5:1 and 1:2 in MIA

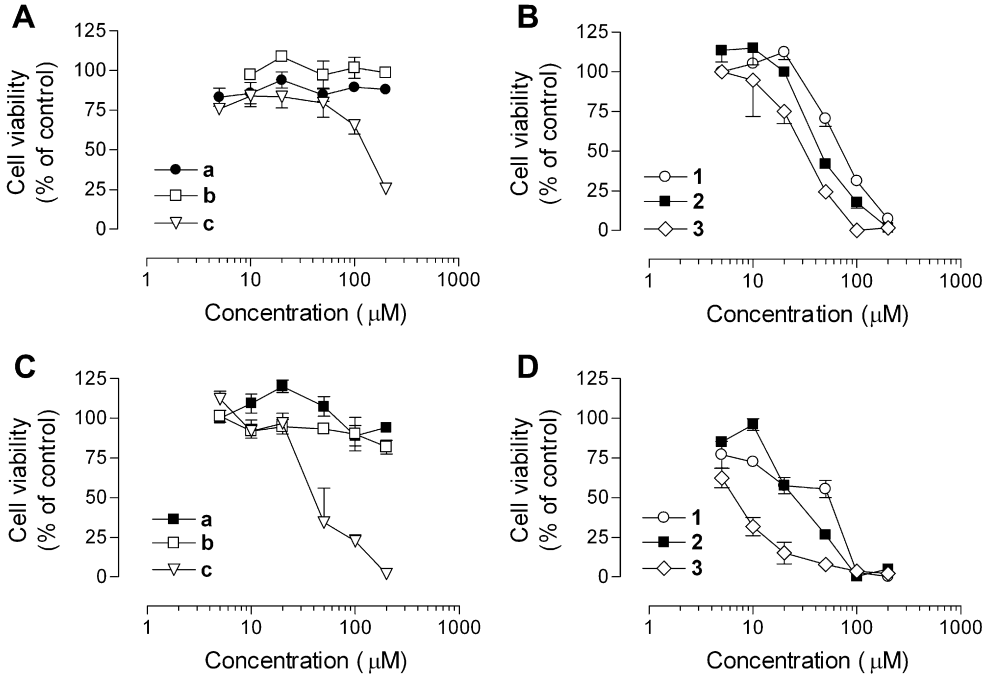


Fig. 2. Concentration-response curves of test compounds in MIA PaCa-2 (A and B) and COLO320 (C and D) cells after 72 h. Data are expressed as mean ± S.E.M ($n = 3$).

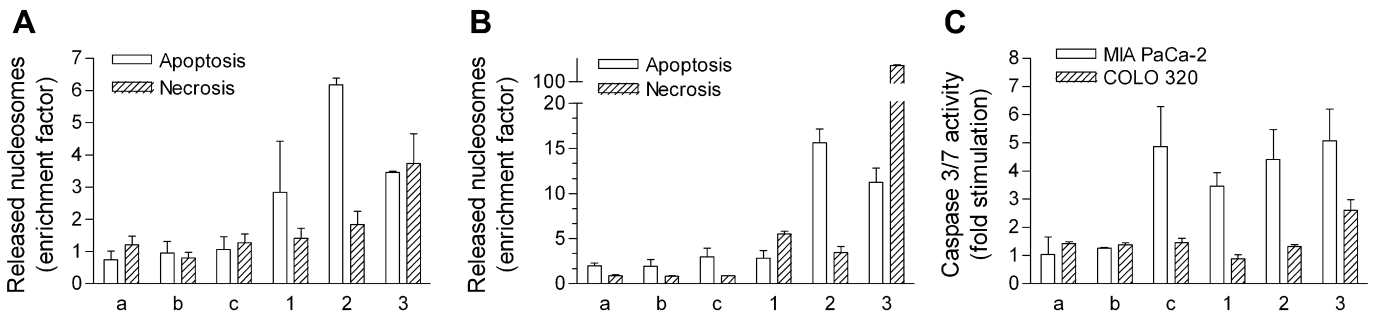


Fig. 3. Apoptotic and necrotic cell death by test compounds 200 μM in MIA PaCa-2 (A) and COLO320 (B) cells after 48 h. Induction of caspase 3/7 activity by test compounds 200 μM in MIA PaCa-2 and COLO320 cells after 24 h (C). Data are expressed as mean ± S.E.M ($n = 3$).

PaCA-2 and COLO320 cells, respectively (Fig. 6B), while caspase 3/7 activity was not affected by treatment (Fig. 6C).

2.2.4. Inhibition of angiogenesis

Growth inhibition studies were performed on HUVEC grown in the presence of the angiogenic factors, bFGF and VEGF. Under this conditions, all test compounds but **a**, significantly inhibited cell proliferation after 96-h exposure ($P < 0.05$) and such an effect occurred at 0.1 μM (Fig. 7A). Sulindac hydroxamic acid (**1**) and its metabolites (**2** and **3**) were more active than their carboxylic acids counterparts and the order of efficacy was **3** > **2** > **1** (Fig. 7A). Cell viability analyses performed by the trypan blue exclusion test indicated that no cytotoxicity occurred in these experimental conditions (data not shown). Finally, high expression levels of both constitutive COX-1 and inducible COX-2 isoforms were demonstrated in HUVEC by RT-PCR (Fig. 7B).

3. Discussion

In the present study, we demonstrated that substitution of the carboxylic acid group with the hydroxamic acid moiety substantially enhances in vitro proapoptotic and antiangiogenic activities of sulindac and sulindac metabolites. Concerning the molecular mechanism of action, hydroxamic acid derivatives were able to promote an increase in the bax/bcl-2 expression ratio and induction of caspase 3/7, two enzymes involved in the effector phase of apoptosis. Of note, such an effect was associated with internucleosomal DNA fragmentation suggesting that, at least in this specific cancer cell type, caspase activation may contribute to the proapoptotic action of compounds. These findings appear to be relevant since bcl-2 overexpression confers resistance to sulindac-induced apoptotic signaling in human colon cancer cells [18].

Growing evidence supports the hypothesis that the development of cancer is angiogenesis-dependent. Beyond its role in the metastatic spread and growth of advanced tumors, angiogenesis could also represent a useful target for chemoprevention since the acquisition of a blood supply is critical for the growth of an early neoplastic lesion [19]. We demonstrated that sulindac hydroxamic acid and metabolites exerted remarkable in vitro antiangiogenic activity. Particularly, hydroxamic acid derivatives of sulindac sulfone and sulfide were able to inhibit 50% of HUVEC growth stimulated by proangiogenic factors at 0.1 μM and such an effect was higher than that observed for sulindac and metabolites.

With regard for the involvement of COX enzymes in the molecular mechanism of action of sulindac hydroxamic acid, it is noteworthy that attaching hydroxamic acid to COX inhibitors did not affect their ability to arrest prostaglandin synthesis [12]. Therefore, we can assume that the hydroxamic acid derivative of

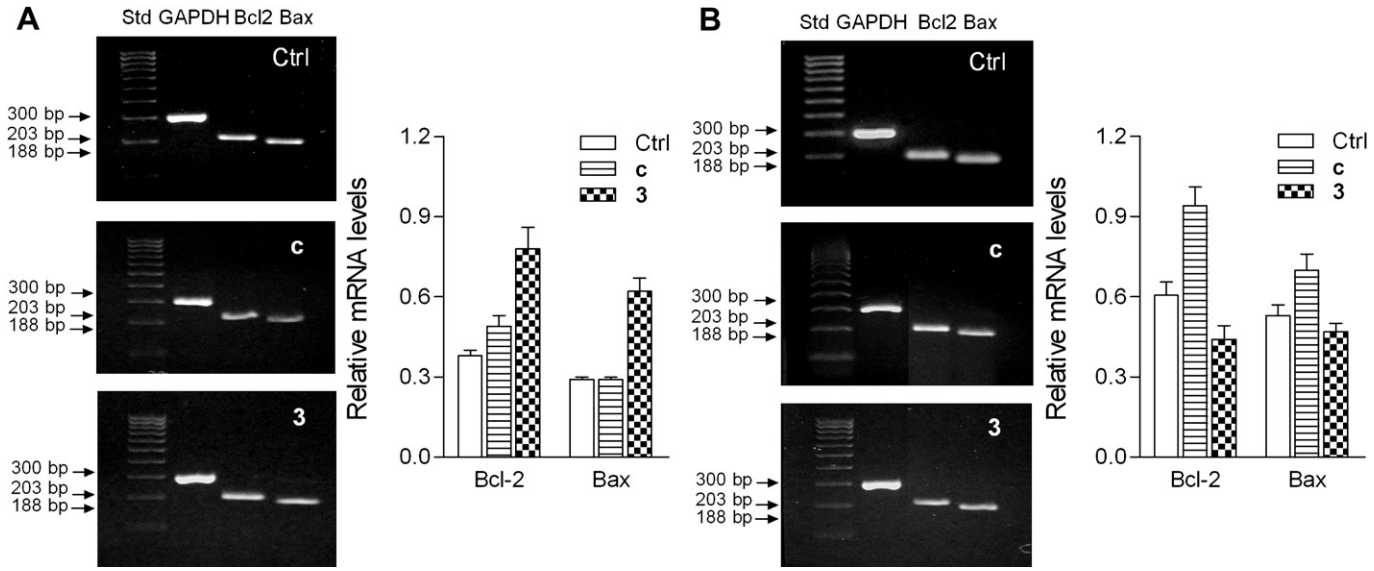


Fig. 4. Effect of **c** and its hydroxamic derivative, **3**, at 200 μM for 24 h on bax and bcl-2 mRNA expression levels in MIA PaCa-2 (A) and COLO320 (B) cells. Levels of mRNA were measured by semi-quantitative RT-PCR, and normalized to GAPDH mRNA. Data are expressed as mean ± S.E.M ($n = 3$).

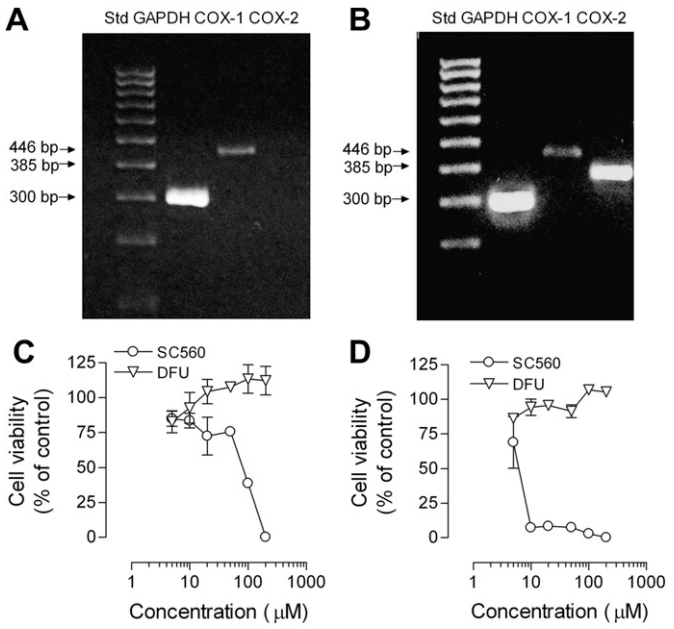


Fig. 5. Expression levels of COX-1 and COX-2 mRNA in MIA PaCa-2 (A) and COLO320 (B) cells. Concentration-response curves of the selective COX inhibitors, SC560 (COX-1) and DFU (COX-2) in MIA PaCa-2 (C) and COLO320 (D) cells after 72 h. Data are expressed as mean ± S.E.M ($n = 3$).

sulindac sulfide, **3**, is able to maintain the COX inhibitor activity. With this in mind, our findings suggest that both COX-dependent and COX-independent effects might contribute to the mechanism of action of test compounds. This conclusion is based on the following lines of evidence: (i) sulindac sulfide (the COX-active metabolite of sulindac) and its hydroxamic acid derivative, **3**, were the most active compounds tested within carboxylic and hydroxamic acids series, respectively; (ii) sulindac sulfide and **3** were active against both COX-2-negative (MIA PACa-2) and COX-2-positive (COLO320) cells; and (iii) selective inhibition of COX-1 but not COX-2 can mimic their effects both in terms of potency and efficacy.

Several lines of evidence indicate that COX-2 isoform and its products are generally highly expressed in premalignant and malignant lesions of intestinal epithelia and they are involved in stimulation of tumor growth and metastases [19]. Our findings demonstrated that COLO320 cells were more sensitive to the cytotoxic action of test compounds than MIA PaCa-2 cells. While the COX-1 isoform was expressed at low levels in both cancer cell lines, differences in COX-2 gene expression were observed. Specifically, COLO320 cells were characterized by high levels of COX-2 mRNA whereas, in line with literature data [20], COX-2 gene was not expressed in MIA PaCa-2 cells. Although these data suggest a possible relationship between COX-2 expression and in vitro drug response, the role of the inducible isoform of COX in the maintenance of the COLO320 phenotype appears to be negligible in our experimental setting since selective COX-2 inhibition did not affect cell proliferation and sulindac sulfide (the COX-active metabolite of sulindac) was also active against MIA PaCa-2 cells. Pancreatic MIA Paca-2 cells were less responsive than COLO320 cells to compound-induced cytotoxicity, a phenotypic feature most probably due to the intrinsic apoptotic resistance of this specific cell type, as previously reported [21]. Therefore, we can speculate that the antiproliferative and proapoptotic effects of the COX-active compounds tested in the present study (sulindac sulfide and its hydroxamic acid derivative, **3**) might be partially mediate by COX-1 inhibition, since the selective COX-1 inhibitor SC560 can mimic their effect, both in terms of potency and efficacy. Evidence in line with this notion was reported on the effect of low-dose aspirin (COX-1 inhibitor) against metachronous polyps and on the significant role of COX-1 polymorphisms in determining the chemopreventive potential of NSAIDs [22].

It has been proposed that a potential COX-mediated mechanism responsible for the tumor suppressive effects of sulindac might result from an accumulation of the prostaglandin precursor arachidonic acid, which stimulates the conversion of sphingomyelin to ceramide, a known mediator of apoptosis [17]. Ceramide, in turn, mediated caspase-independent programmed cell death [23]. Although we did not directly measure changes in intracellular arachidonic acid concentration following treatment with **3**, exogenous arachidonic acid induced cytotoxicity and caused apoptosis in colon and pancreatic cancer cells, independently from caspase 3/7 activation. As previously demonstrated for sulindac sulfide in human colon cancer cells [17], our findings suggest that COX-1 inhibition and the consequent arachidonic acid increase might partially account for **3** cytotoxicity.

With regard for the clinical relevance of our results, we demonstrate that the cytotoxic activity against pancreatic and colon cancer cells and the in vitro antiangiogenic effect induced by sulindac hydroxamic acid and metabolites occur at concentrations lower than carboxylic acid counterparts (i.e., in the low micromolar range). Plasma levels of sulindac have been reported to be up to 50 μM after oral administration and the concentration of its sulfide metabolite is higher in the intestines than in other organs, due to the enterohepatic circulation and to the metabolic activation of parent drug by gut flora [24]. Therefore it is conceivable that sulindac hydroxamic acid and metabolites can reach a threshold level required for biologically activity in the tumor microenvironment, in vivo.

In conclusion, the chemical transformation of sulindac into hydroxamic acid derivative enhances its proapoptotic and antiangiogenic properties, thus improving the therapeutic potential of the drug against human cancer.

Table 2
IC_{50s} (μM, mean ± SEM; $n = 3$) of selective COX inhibitors on human cancer cell lines.

Compounds	Cell line	
	MIA PaCa-2	COLO320
SC560	102 ± 1.2	5.6 ± 1.1[a]
DFU	>200	>200

SC560: selective COX-1 inhibitor; DFU: selective COX-2 inhibitor.
[a] Changes in the mean IC50 values obtained for each cell line that are significant ($p < 0.001$; Student's t-test).

4. Experimental

4.1. Chemistry

4.1.1. Materials and physical measurements

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Infrared (IR) spectra for comparison of compounds were recorded on a Mattson 1000 FTIR spectrometer. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini 200 (200 MHz) in a ca. 2% solution of $CDCl_3$ for all compounds. Peak positions are given in parts per million (ppm, δ units). The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. Reactions were routinely monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (Merck $60F_{254}$) and hydroxamic acids were visualized with $FeCl_3$ aqueous solution. Flash chromatography was carried out through

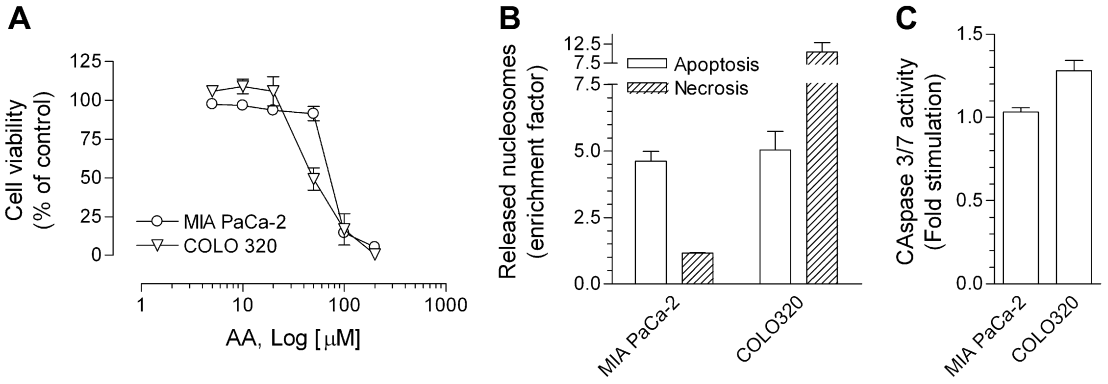


Fig. 6. Cytotoxicity (A), apoptosis/necrosis (B) and caspase 3/7 induction (C) after treatment of human cancer cells with arachidonic acid (AA) 200 μM for 72, 48 and 24 h, respectively. Data are expressed as mean ± S.E.M ($n = 3$).

glass columns containing 40–63 μm silica gel (Macherey–Nagel Silica Gel 60). Solvents and reagents were obtained from commercial sources in the appropriate grade and were used without further purification unless otherwise indicated. Reactions were run under an argon atmosphere. Elemental analyses were carried out by our analytical laboratory and were consistent with theoretical values to within ±0.4%.

4.1.2. Procedures for the synthesis of sulindac metabolites

*4.1.2.1. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylsulfonyl)benzylidene)-3H-inden-1-yl)acetic acid (**b**).* A solution of oxone (1.7 mmol) in H_2O (3 ml), was added portionwise to stirred and cooled (0 °C) solution of sulindac (**a**) (1.4 mmol) in THF/MeOH 1/1 v/v (10 ml). After stirring at room temperature for 24 h, the mixture was extracted with AcOEt, washed with water (20 ml) and the organic phase was dried and evaporated in vacuo to yield the corresponding sulfone derivative, **b**. Compound **b** was yellow solid, mp: 199–200 °C, yield 75%; ^{1}H NMR ($CDCl_3$, δ ppm): 8.03–7.98 (d, $J = 8.2$ Hz, 2H); 7.72–7.67 (d, $J = 8.2$ Hz, 2H); 7.14–7.06 (m, 2H); 6.91–6.86 (m, 1H); 6.62–6.52 (m, 1H) 3.60 (s, 2H); 3.13 (s, 3H); 2.21 (s, 3H). Anal. Calcd for $C_{19}H_{17}FO_4S$: C, 63.32, H, 4.75. Found: C, 63.17; H, 3.58.

*4.1.2.2. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylthio)benzylidene)-3H-inden-1-yl)acetic acid (**c**).* To a solution of **a** (0.20 mmol) and $TiCl_4$ (0.3 mmol) in THF (2.5 ml) was added a solution of triphenylphosphine (0.24 mmol) in THF (1.5 ml) and the mixture was stirred at appropriate temperature under an argon atmosphere. The reaction was quenched with saturated sodium hydrogen carbonate (10 ml), and the mixture was extracted with ether (3 × 10 ml). The combined ether layers were washed with brine (1 × 10 ml) and dried over Na_2SO_4. This organic layer was filtered and evaporated under reduced pressure, and then the crude product was triturated with Et_2O to remove the excess of triphenylphosphine, filtered and evaporated in vacuo to yield a crude product that was purified by titration with Et_2O to give the sulfone **c** as a yellow solid. Compound **c**: mp: 186–190 °C; yield 75%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.73–7.30 (m, 4H); 7.14 (s, 2H); 6.91–6.86 (m, 1H); 6.63–6.53 (m, 1H); 3.59 (s, 2H); 2.54 (s, 3H); 2.20 (s, 3H). Anal. Calcd for $C_{20}H_{17}FO_2S$: C, 70.57; H, 5.03. Found: C, 70.76; H, 5.24.

*4.1.3. General procedure for the synthesis of O-TBDMS acid hydroxyamides **4**–**6***

1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (EDCI) was added portionwise (1 mmol) to stirred and cooled (0 °C) solution of the carboxylic acids **a**–**c** (1 mmol) in dry CH_2Cl_2 (18 ml). After stirring at room temperature for 20 h, the mixture was washed with water (20 ml) and the organic phase was dried and evaporated in vacuo. The residue was purified by flash chromatography to yield pure silyl esters **4**–**6** as oils.

*4.1.3.1. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylsulfinyl)benzylidene)-3H-inden-1-yl) acetic acid (tert-butyldimethylsilyl)hydroxyamide (**4**).* The title compound was prepared from carboxylic acid **a** following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane-ethyl acetate 4/6 v/v to give **4** as a yellow oil, yield 40%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.71–7.68 (m, 4H); 7.18 (s, 2H); 6.87–6.83 (m, 1H); 6.63–6.54 (m, 1H); 3.51 (s, 2H); 2.81 (s, 3H); 2.21 (s, 3H); 0.91 (s, 9H); 0.14 (s, 6H). Anal. Calcd for $C_{25}H_{32}FNO_3SSi$: C, 63.40; H, 6.82; N, 2.96. Found: C, 63.57; H, 6.98; N, 3.14.

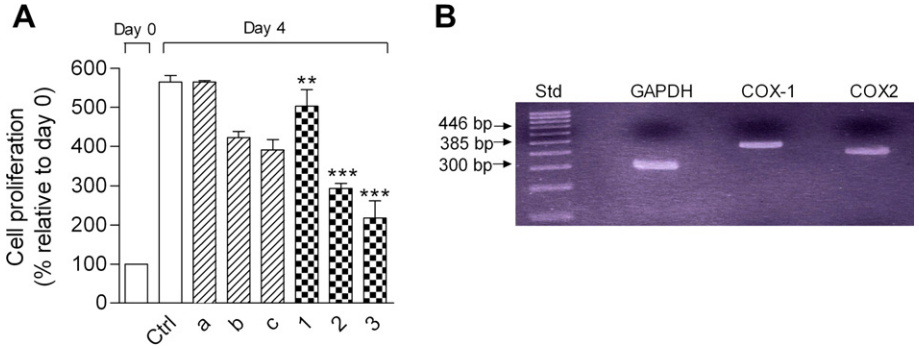


Fig. 7. Effect of test compounds on HUVEC proliferation after 96-h exposure at 0.1 μM (A) and expression levels of COX-1 and COX-2 mRNA (B). Data are expressed as mean ± SEM ($n = 3$). $^{**}P < 0.01$ (a *versus* 1), $^{***}P < 0.001$ (**b** *versus* **2** and **c** *versus* **3**).

*4.1.3.2. (E)-2-(6-Fluoro-2-methyl-3-(4-(methylsulfonyl)benzylidene)-3H-inden-1-yl) acetic acid (tert-butyldimethylsilyl)hydroxyamide (**5**).* The title compound was prepared from carboxylic acid **a** following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane-ethyl acetate 4/6 v/v to give **5** as a yellow oil, yield 40%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.97–7.93 (m, 2H); 7.72–7.68 (m, 2H); 7.19–7.10 (m, 2H); 6.94–6.83 (m, 1H); 3.58 (s, 2H); 3.11 (s, 3H); 2.21 (s, 3H); 0.91 (s, 9H); 0.14 (s, 6H). Anal. Calcd for $C_{25}H_{32}FNO_4SSi$: C, 61.32; H, 6.59; N, 2.86. Found: C, 61.13; H, 6.42; N, 2.70.

*4.1.3.3. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylthio)benzylidene)-3H-inden-1-yl) acetic acid (tert-butyldimethylsilyl)hydroxyamide (**6**).* The title compound was prepared from carboxylic acid **a** following the general procedure. The crude product was purified by flash chromatography on silica gel using hexane-ethyl acetate 4/6 v/v to give **6** as a yellow oil, yield 40%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.71–7.30 (m, 4H); 7.15 (s, 2H); 6.89–6.83 (m, 1H); 6.63–6.60 (m, 1H); 3.51 (s, 2H); 2.55 (s, 3H); 2.21 (s, 3H); 0.91 (s, 9H); 0.14 (s, 6H). Anal. Calcd for C26H32FNO$_2$SSi: C, 66.49; H, 6.87; N, 2.98. Found: C, 66.68; H, 7.10; N, 3.16.

*4.1.4. General procedure for the preparation of acid hydroxyamides **1**–**3***

Trifluoroacetic acid (4.4 mL, 57 mmol) was added dropwise to a stirred and cooled solution (0 °C) of 4–6 (1 mmol) in dry CH_2Cl_2. The solution was stirred under these reaction conditions for 5 h and the solvent was removed in vacuo to give the hydroxyacetamide derivatives (**1**–**3**).

*4.1.4.1. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylsulfinyl)benzylidene)-3H-inden-1-yl)-N (**1**).* The title compound was prepared from **4** following the general procedure. The crude product was purified by titration with Et_2O to give **1** as yellow solid, mp: 208–209 °C; yield 70%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.76–7.64 (m, 4H); 7.21–7.15 (m, 2H); 6.87–6.81 (m, 1H); 6.65–6.57 (m, 1H); 3.58 (s, 2H); 2.82 (s, 3H); 2.22 (s, 3H). Anal. Calcd for $C_{20}H_{18}FNO_3S$: C, 64.68; H, 4.88; N, 3.77. Found: C, 64.87; H, 5.12; N, 3.96.

*4.1.4.2. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylsulfonyl)benzylidene)-3H-inden-1-yl)-N-hydroxyacetamide (**2**).* The title compound was prepared from **5** following the general procedure. The crude product was purified by titration with Et_2O to give **2** as yellow solid; mp: 210–215 °C; yield 70%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.97–7.93 (m, 2H); 7.72–7.68 (m, 2H); 7.19–7.10 (m, 2H); 6.94–6.83 (m, 1H); 3.60 (s, 2H); 3.11 (s, 3H); 2.22 (s, 3H). Anal. Calcd for $C_{20}H_{18}FNO_4S$: C, 62.00; H, 4.68; N, 3.62. Found: C, 61.83; H, 4.81; N, 3.75.

*4.1.4.3. (Z)-2-(6-Fluoro-2-methyl-3-(4-(methylthio)benzylidene)-3H-inden-1-yl)-N-hydroxyacetamide (**3**).* The title compound was prepared from **6** following the general procedure. The crude product was purified by titration with Et_2O to give **3** as yellow solid, mp: 199–201 °C; yield 70%; ^{1}H NMR ($CDCl_3$, δ ppm): 7.46–7.30 (m, 4H); 7.18 (s, 2H); 6.86–6.81 (m, 1H); 6.65–6.57 (m, 1H); 3.54 (s, 2H); 2.55 (s, 3H); 2.19 (s, 3H). Anal. Calcd for $C_{20}H_{18}FNO_2S$: C, 67.59; H, 5.10; N, 3.94. Found: C, 67.73; H, 5.23; N, 4.12.

4.2. Pharmacology

4.2.1. Cell culture conditions

The human cancer cell lines MIA PaCa-2 (pancreas) and COLO320 (colon) (American Type Culture Collection, Manassas, VA), were cultured in DMEM medium supplemented with L-glutamine (2 mM), 10% fetal bovine serum, 2.5% horse serum, 50 IU/ml penicillin and 50 μg/ml streptomycin (Sigma–Aldrich, Milano, Italy) at 37 °C in an atmosphere of 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were cultured in an optimized media containing the angiogenic factors, bFGF and VEGF (Lonza, Walkersville, MD, USA).

4.2.2. Cell viability assay

Pancreatic or colon cancer cells (2–5 × 10^3/well) were seeded into 96-well microtiter plates and received compounds from 5 to 200 μM for 72 h. Following drug exposure, cell viability was assessed using a method based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenase activity (Cell proliferation reagent WST-1; Roche, Mannheim, Germany). Inhibition of proliferation was reported as the percentage reduction of UV absorbance of treated cells *versus* control cultures and the concentration of compounds that decreased cell viability by 50% (IC_{50}) was calculated by non-linear least squares curve fitting (GraphPad Software, San Diego, CA, USA). DMSO concentration in the culture medium never exceeded 0.2%.

4.2.3. Caspase activity assay

Enzyme activity was assayed by the Apo-ONE™ Homogeneous Caspase 3/7 assay (Promega, Madison, WI, USA). Briefly, cells were seeded at 5 × 10^3/well and treated with compounds at 100 μM for 24 h. Subsequently, the Caspase 3/7 assay substrate was added and the fluorescence was measured by spectrofluorimeter at excitation and emission wavelengths of 485 and 530 nm, respectively. Values were expressed as ratio between fluorescent signals generated in cells treated with compounds and those produced in untreated cells (vehicle alone).

4.2.4. DNA fragmentation assay

Cells were treated for 24 or 48 h with tested compounds at 200 μM, harvested by trypsinization and combined with detached cells. Apoptosis was then assessed by the Cell Death Detection ELISA kit (Roche, Mannheim, Germany) based on the recognition of released nucleosomes after DNA internucleosomal fragmentation by a mouse monoclonal antibody directed against DNA and histones. The assay allowed the specific determination of mono- and oligo-nucleosomes in the cytoplasmic (apoptosis) or in the supernatant fraction (necrosis) of cell lysates.

4.2.5. RT-PCR assay

RNA from cells was extracted by using the RNeasy Mini kit and reverse-transcribed by the QuantiTect Reverse Transcription kit. PCR was performed by the Hot StartTaq Master Mix kit. Primers used were: 5′-GCCTGACTCCTTCAAGATCG-3′ (F) and 5′-AGGGACAGGTCTTGGTGTTG-3′ (R) for COX-1; 5′-TGAAACCCACTCCAAACACA-3′ (F) and 5′-AACTGATGCGTGAAGTGCTG-3′ (R) for COX-2; 5′-TCTGACGGCAACTTCAACTG-3′ (F) and 5′-TTGAGGAGTCTCACCCAACC-3′ (R) for Bax; 5′-TCCATGTCTTTGGACAACCA-3′ (F) and 5′-CTCCACCAGTGTTCCCATCT-3′ (R) for Bcl-2; 5′-GTGAAGGTCGGAGTCAACG-3′ (F) and 5′-GGTGAAGACGGCCAGTGGACTC-3′ (R) for GAPDH and the expected amplification products were 446, 385, 188, 203 and 300 bp long, respectively. Relative densitometry of bands was measured using NIH ImageJ gel analysis.

4.2.6. Anti-angiogenic activity

HUVEC were sub-cultured into twenty-four-well plates at a density of 10^4 cells/well over night and kept in serum-free medium for 24 h. Cells were then incubated in growth medium in the presence or absence of 0.1 μM test compounds for 96 h exposure. At the end of the experiments, cells were harvested and counted by haemocytometer and changes in cell growth were expressed as a percentage relative to day 0.

4.2.7. Data analysis and statistical procedures

Data were expressed as mean ± standard error of the mean (SEM) of three measurements. Data analysis was performed with GraphPad Prism software. The 50% inhibitory concentration of cell growth (IC50) was calculated with a non-linear least squares curve

using a sigmoid dose–response (variable slope) equation. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparison. $P < 0.05$ was taken as level of significance.

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