NO-sulindac inhibits the hypoxia response of PC-3 prostate cancer cells via the Akt signalling pathway

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Nitric oxide-donating non-steroidal anti-inflammatory drugs are safer than traditional NSAIDs and inhibit the growth of prostate cancer cells with greater potency than NSAIDs. In vivo, prostate cancer deposits are found in a hypoxic environment which induces resistance to chemotherapy. The aim of this study was to assess the effects and mechanism of action of a NO-NSAID called NO-sulindac on the PC-3 prostate cancer cell line under hypoxic conditions. NO-sulindac was found to have pro-apoptotic, cytotoxic, and anti-invasive effect on PC-3 cells under normoxia and hypoxia. NO-sulindac was significantly more cytotoxic than sulindac at all oxygen levels. The sulindac/linker and NO-releasing subunits both contributed to the cytotoxic effects of NO-sulindac. Resistance of PC-3 cells to NO-sulindac was induced as the oxygen concentration declined. Hypoxia-induced chemoresistance was reversed by knocking-down hypoxia-inducible factor-1α (HIF-1α) mRNA using RNAi. Nuclear HIF-1α levels were upregulated at 0.2% oxygen but reduced by treatment with NO-sulindac, as was Akt phosphorylation. NO-sulindac treatment of hypoxic PC-3 cells transfected with a reporter construct, downregulated activation of the hypoxia response element (HRE) promoter. Co-transfection of PC-3 cells with the HRE reporter construct and myristoylated Akt (constitutively active Akt) plasmids reversed the NO-sulindac induced reduction in HRE activity. Real-time polymerase chain reaction analysis of hypoxic, NO-sulindac treated PC-3 cells showed downregulation of lysyl oxidase and carbonic anhydrase IX mRNA expression. Collectively, these novel findings demonstrate that NO-sulindac directly inhibits the hypoxia response of PC-3 prostate cancer cells by inhibiting HIF-1α translation via the Akt signalling pathway. The ability of NO-sulindac to inhibit tumour adaption to hypoxia has considerable relevance to the future management of prostate cancer with the same cellular properties as PC-3.

Key words: hypoxia; prostate cancer; nitric oxide donors; NO-NSAIDs; Akt

In Western Europe and North America, prostate cancer is the commonest cancer and the second most common cause of cancer death in men. In the United Kingdom, prostate cancer accounts for 23% of all new male cancer diagnoses and 13% of male cancer-related deaths.1Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit tumourigenesis in a variety of cancers.2,3 In vitro studies have shown that conventional NSAIDs, at physiological achievable doses, inhibit the proliferation of immortalised human prostate cancer cells.4 Additionally, meta-analyses of observational studies of men taking regular NSAIDs have reported statistically significant reductions in risk of prostate cancer.5,6 However, conventional NSAIDs have several side-effects, particularly gastrointestinal bleeding, which limit their use in elderly patients with prostate cancer. In an attempt to reduce the side-effects of traditional NSAIDs, cyclooxygenase-2 inhibitors and nitric oxide (NO)ₕ-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) have been developed. NO-NSAIDs consist of a traditional NSAID group to which a NO-donating component has been covalently attached via an aromatic or aliphatic spacer. The NOₕ group of NO-NSAIDs confers its protective effect on the gastric mucosa by increasing mucosal blood supply and mucous secretion. The increased mucous secretion protects patients from the most serious side effect of NSAIDs, namely, gastric erosions.9 Therefore, NO-NSAIDs combine the anti-proliferative effects of NSAIDs with the gastric protection10,11 and potential tumouricidal effects of NO.12 Thus, NO-NSAIDs are potentially powerful agents against malignancy.

Treatment of human prostate, prostate, lung, colon, and tongue carcinoma cell lines with NO-NSAIDs has shown inhibition of cell proliferation, induction of apoptosis and altered cell cycle distribution.13 In vivo colorectal cancer models have also shown an anti-proliferative effect for NO-aspirin.14 Furthermore, NO-NSAIDs have been demonstrated to have chemopreventative activity in both colorectal and pancreatic cancer models.15,16 Hypoxia has been demonstrated in many solid human tumours, including prostate cancer.17-19 Hypoxia is an independent prognostic indicator of poor clinical outcome for patients with prostate and other cancers.20,21 Hypoxia has also been shown to correlate with increased tumour invasiveness, metastases and resistance to some chemotherapy treatments in prostate cancer.20,22-24

The master regulator of oxygen homeostasis is the transcription factor hypoxia-inducible factor-1 (HIF-1).22 HIF-1, an αβ heterodimeric transcription factor, consists of a constitutively expressed HIF-1α subunit and a hypoxia-inducible HIF-1α subunit. There are 5 groups of HIF-1 target genes particularly relevant to tumourigenesis, including angiogenic factors [e.g., vascular endothelial growth factor (VEGF)], glucose transporters, glycolytic enzymes, survival factors and invasion factors.20 HIF-1 is at the centre of most adaptive responses of cancer cells to hypoxia and overexpression of HIF-1α has been associated with increased patient mortality in several cancer types.25,26 Understanding the regulation of factors, such as HIF-1α, which modulate the response of a tumour to hypoxia may be helpful in designing anti-cancer therapies.

The aims of this study were to: (a) assess, for the first time, the effect of a NO-NSAID called NO-sulindac on the survival and invasive potential of PC-3 hormone-insensitive prostate cancer cell line under hypoxic conditions; (b) determine the effect that NO-sulindac had on the hypoxia response mounted by prostate tumour cells to low oxygen conditions; and (c) identify possible mechanisms of action.

Abbreviations: CAIX, carbonic anhydrase IX; COX-2, cyclooxygenase-2; DAPI, 4′,6-diamino-2-phenylindole; FITC, fluorescein isothiocyanate; GLUT-1, glucose transporter-1; HBSS, Hank’s Buffered Salt Solution; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia response element; LOX, lysyl oxidase; mAb, monoclonal antibody; NO-NSAIDs, nitric oxide-donating non-steroidal anti-inflammatory drugs; NOₕ, nitric oxide; NP40, Nonidet P40; NSAIDs, non-steroidal anti-inflammatory drugs; NSS, nuclear staining solution; PCR, polymerase chain reaction; PI, propidium iodide; RT, reverse transcription; SD, standard deviation; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor.

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One millilitre of 1 mg/ml RNase A solution (diluted with sodium citrate and 0.3 ml Nonidet P40 (NP40), in 100 ml distilled water). One millilitre of 1 mg/ml RNase A solution (diluted with sodium citrate and 0.3 ml Nonidet P40 (NP40), in 100 ml distilled water). One millilitre of 1 mg/ml RNase A solution (diluted with sodium citrate and 0.3 ml Nonidet P40 (NP40), in 100 ml distilled water). One millilitre of 1 mg/ml RNase A solution (diluted with sodium citrate and 0.3 ml Nonidet P40 (NP40), in 100 ml distilled water).

**Figure 1** – Chemical structures of traditional sulindac, its NO-derivative (NO-sulindac (NCX 1102)), and the denitrated analogue of NO-sulindac (NCX 221).

**Material and methods**  
**Cell culture and reagents**
All chemicals and reagents were purchased from Sigma (Gillingham, UK) unless stated otherwise. PC-3 cells (obtained from the European Collection of Cell Cultures, Salisbury, UK) were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 2 mmol glutamine (all Invitrogen, Paisley, UK). Cells were seeded into 6-well plates at 3 x 10^5 cells/well for all experiments, except where stated. Hypoxia was induced by incubating PC-3 cells for 48 hr within a humidified hypoxia incubator at 0.2% oxygen using a PROOX 110 oxygen controller (BioSpherix, Redfield, NY). NO-sulindac (NCX 1102), NCX 221 denitrated analogue and sulindac (Fig. 1) were donated by NicOx (Sophia Antipolis, France) and prepared in dimethyl sulfoxide (DMSO) with final concentrations of ≤0.1%.

**Assessment of cell death**
Assessment of cytotoxicity was performed using propidium iodide (PI) as described previously. Apoptosis was assessed using annexin V/PI staining (all reagents Bender Medsystems, Vienna, Austria) followed by flow cytometric analysis. After washing cells in D-PBS, 5 µl annexin V-fluorescein isothiocyanate (FITC) was added to 195 µl D-PBS diluted cells and incubated in the dark at room temperature for 10 min. Cells were then washed and resuspended in 190 µl binding buffer (diluted 1:4 in distilled water). Ten microlitres of PI (20 µg/ml) was added before flow cytometric analysis. The emission wavelength of PI was plotted against the emission wavelength of annexin V-FITC. Early apoptotic cells were annexin V positive, PI negative; late apoptotic/early necrotic cells were annexin V positive, PI positive; and necrotic cells were annexin V negative, PI positive. Very few cells (<1%) stained annexin positive/PI negative following NO-sulindac treatment, i.e., were not identified as dying/dead by PI staining alone. As such, it was elected to use PI staining alone for determination of cytotoxic effect in the majority of experiments.

**Estimation of nitrite production**
Nitrite production, an indicator of the production of nitric oxide, was measured using the Griess reagent (1% sulfanilamide and 0.1% (N-1-naphthyl) ethylenediamine dihydrochloride) in 2.5% w/w phosphoric acid. Griess reagent (25 µl) and medium from treated cells (75 µl) were mixed and incubated for 10 min at room temperature in a 96-well plate. Absorbance was measured at 540 nm using a MR3000 Dynatech plate reader (Dynex Technologies, Worthing, UK) and compared to a sodium nitrite standard curve (concentration range 0.024–50 µM).

**Cell cycle analysis**
PC-3 cells were harvested as mentioned earlier and resuspended in 1 ml of nuclear staining solution (NSS) (NSS: 5 mg PI, 100 mg sodium citrate and 0.3 ml Nonidet P40 (NP40), in 100 ml distilled water). One millilitre of 1 mg/ml RNase A solution (diluted with 1.12% sodium citrate) was then added to the PC-3 cells and incubated in the dark at room temperature for 30 min. The DNA content of the cells was then analysed by flow cytometry.

**Assessment of cell invasion**
BD BioCoat™ Tumor Invasion System (BD Biosciences, San Jose, CA) was used to assess cell invasion. PC-3 cells were seeded at 1 x 10^5 cells/well in RPMI-1640 medium without FCS. RPMI-1640 medium with 10% FCS was used as the chemottractant in the lower chamber. After 24 hr incubation under standard conditions, the cells were treated as per experimental protocol.

Cell invasion was assessed by measuring the fluorescence of invading cells labelled with 4 µg/ml Calcein AM (Molecular Probes, Eugene, OR) in Hank’s Buffered Salt Solution (HBSS; Invitrogen). Fluorescence of invading cells was determined using a fluorescent plate reader with bottom reading capabilities at excitation/emission wavelengths of 485/530 nm (Synergy HT multimode microplate reader, BioTek, Winooski, VT).

**Western blot analysis**
Western blot analysis, using nuclear protein extracts, was performed as described previously. Equal amounts of nuclear protein (10 µg) were subjected to SDS-PAGE in 7.5% gels. Proteins were electrophoresed and then transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary and secondary antibodies, and subjected to chemiluminescence detection and autoradiography. β-actin mAb (Abcam, Cambridge, UK) at 1:10,000 was used as a loading control for all western blots. HIF-1α mAb (BD Transduction Laboratories, Oxford, UK) was used at 1:250 dilution. An anti-mouse HRP conjugated secondary antibody (Upstate, Milton Keynes, UK) at 1:5,000 was used with β-actin and HIF-1α mAb. Phospho-Akt (Ser473) mAb (Invitrogen) was used at 1:1,000 dilution with an anti-rabbit HRP linked secondary antibody (Cell Signaling Technologies, Danvers, MA) at 1:2,000.

**Immunocytochemical staining**
Immunocytochemistry and subsequent fluorescent microscopy and image capture were performed as previously described. PC-3 cells were grown on coverslips until 60–80% confluent and then treated as per experimental protocol. Cells were fixed and immunocytochemistry performed. The primary antibodies used at 1:100 in 10% donkey serum were HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA) and fibrillarin (Cytoskeleton, Denver, CO). The FITC- and Texas red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:200 in 1.5% donkey serum, cells were mounted using Vectashield (Vector Laboratories, Peterborough, UK) at 1:5,000.
VT). Each channel was recorded independently, and pseudocolour images were superimposed. Images were captured with single emission filters and analysed using IPLab Spectrum 3.6 (Scanco-lytics Corp., Fairfax, VA).

Luciferase assays

Transient transfections with the pGL2-Tk-HRE promoter reporter construct (Dr. G Melillo, Frederick, MD) were performed using Fugene reagent (Roche Applied Science, Lewes, UK) at a ratio of 3 μg Fugene per 1 μg plasmid DNA. PC-3 cells were plated out in 6-well plates (2.5 × 10^5 cells/well) and transfected after 24 hr. Cells transfected with 0.5 μg were co-transfected with 0.5 μg of the reporter construct. Forty-eight hours after transfection, the cells underwent drug treatment ± hypoxia. After a further 48 hr, the cells were lysed using reporter lysis buffer (Promega). To quantify luciferase activity, 20 μl lysate was combined with 20 μl luciferase assay reagent (Promega) and the resulting light emission measured immediately using a luminometer (Fluoroskan Ascent FL, Thermo Electron, Basingstoke, UK). To measure β-galactosidase activity, 50 μl cell lysate was combined with 50 μl β-galactosidase assay 2X buffer (Promega). Following incubation at 37°C for 3 hr and stopping the reaction with 150 μl 1 M sodium carbonate, the absorbance of the samples was read at 420 nm using a MR3000 Dynatech plate reader.

RNA extraction and reverse transcription

RNA extraction was performed as previously described.27,31 One microgram of total RNA was used for reverse transcription (RT), after treatment with RQ1 DNase (Promega). RT was performed using AMV reverse transcriptase (Promega) and random primers (Promega). Following incubation for 10 min at room temperature, the following RT program was used: 42°C for 1 hr and 99°C for 5 min, before cooling to 4°C using a PCR Sprint Thermo Cycler (Thermo Electron Corporation, Basingstoke, UK).

Real time PCR

Real-time polymerase chain reaction (PCR) was performed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Eukaryotic 18S rRNA endogenous control (VIC/TAMRA probe, primer limited) and TaqMan Gene Expression Assays were purchased from Applied Biosystems. The relative standard curve method was used to determine the fold change in gene expression between treated and untreated cells. The sulindac/linker and NO-releasing subunits are responsible for the cytotoxic effect of NO-sulindac

Experiments were performed to determine the role of each subunit of NO-sulindac (sulindac, linker/spacer and NO-releasing group; Fig. 1) in the cytotoxic effect of NO-sulindac. After 48 hr exposure of PC-3 cell to DMSO or sulindac under normoxia, the nitrite concentration, used as estimate of nitric oxide production, was below the limits of detection of the Griess assay (Fig. 2b). Furthermore, the nitrite concentration following treatment of PC-3 cells at both 25 μM and 50 μM NO-sulindac for 48 hr was measurable but low (<2 μM). The nitrite concentration of sodium nitroprusside (SNP) combined in equimolar amounts with sulindac was 20- to 32-fold greater than that of NO-sulindac. Despite this finding, the combined sulindac + SNP treatment did not match the cytotoxic effect of NO-sulindac and was not significantly more cytotoxic than DMSO control treated cells (Fig. 2b). However, treatment of PC-3 cells for 48 hr with the denitrated analogue of NO-sulindac, NCX 221 (composed of sulindac and the linker), resulted in a significant reduction in cell survival relative to control treated cells, but did not reduce cell survival to the same extent as NO-sulindac (Fig. 2b). These results suggest that the linker/spacer of NO-sulindac is partly responsible for the cytotoxic effects observed earlier.

Effect of hypoxia on the cytotoxic effect of NO-sulindac

PC-3 cells were incubated at several different oxygen levels (0.2, 5, 10 and 21% oxygen) to assess the effect of hypoxia on cell growth and cytotoxic effects of NO-sulindac. Figures 2c and 2d show the relative survival of PC-3 cells incubated at differing oxygen levels and treated with 25 μM and 50 μM sulindac or NO-sulindac, compared to DMSO vehicle control.

As the cell incubation oxygen level was reduced, the relative survival of PC-3 cells also serially declined (Figs. 2c and 2d). PC-3 cells treated with the DMSO vehicle control incubated at 21% oxygen had a significantly higher relative survival than PC-3 cells incubated at 10.5 or 0.2% oxygen (p < 0.005, Mann-Whitney U test).

At all oxygen levels used, treatment of PC-3 cells with either 25 μM or 50 μM NO-sulindac resulted in a significant reduction in relative survival compared with the DMSO vehicle control at the same oxygen level (Fig. 2c). Conversely, treatment of the PC-3 cells with 25 μM or 50 μM sulindac did not result in a significant difference in relative survival compared to the control, at any oxygen level utilised. At each oxygen level, NO-sulindac was signifi-
significantly more cytotoxic than sulindac ($p < 0.04$, Mann-Whitney U test). However, at both the 25 $\mu$M and 50 $\mu$M doses of NO-sulindac, there appeared to be a reduction in cell death and a relative increase in survival as the oxygen level was reduced.

**NO-sulindac treatment but not hypoxia alone alters the PC-3 cell cycle**

Cell cycle analysis was performed to determine if hypoxia altered the cell cycle of PC-3 cells and was potentially responsible for the reduced cytotoxic effect of NO-sulindac under hypoxia. Figure 3a illustrates that incubation of PC-3 cells under hypoxia and/or treatment with 25 $\mu$M sulindac for 48 hr did not alter the cell cycle other than a doubling of cells at the sub-$G_1$ phase found under normoxia. However, when the cells were treated for 48 hr with 25 $\mu$M NO-sulindac under both normoxia and hypoxia there was an alteration in the cell cycle. At 21% and 0.2% oxygen levels, NO-sulindac resulted in 20.4% and 18.5% apoptosis (sub-$G_1$), respectively, with a corresponding reduction in cells in $G_0/G_1$. Following treatment with NO-sulindac there was an increase in cells in $G_2/M$ from 33.3 to 50.4% at normoxia and from 39.9 to 56.1% under hypoxia.

**NO-sulindac inhibits the invasion of PC-3 cells under normoxia and hypoxia**

Figures 3b and 3c illustrates that NO-sulindac treated PC-3 cells, under both normoxia and hypoxia, underwent a significantly lower level of cell invasion than either sulindac or control treated cells.

Following exposure of normoxic PC-3 cells to 25 $\mu$M NO-sulindac for 24 hr, 24% cells were killed relative to DMSO vehicle control (Fig. 2a). Under the same conditions, 41% of PC-3 cells invaded through the Matrigel of the tumour invasion system. As such, of the 59% PC-3 cells not undergoing invasion, following NO-sulindac treatment, 24% may be due to cell death but at least 35% cells were alive but prevented from invading by NO-sulindac treatment.

**NO-sulindac reduces nuclear HIF-1$\alpha$ protein expression**

Figures 4a and 4b show western blots for HIF-1$\alpha$ at 4 different oxygen levels. Figure 4 shows that HIF-1$\alpha$ was not upregulated at 10 or 5% oxygen level compared to PC-3 cells incubated at 21% oxygen. However, at 0.2% oxygen there was increased HIF-1$\alpha$ in the nucleus. At each of the hypoxic oxygen levels, there was a downregulation of HIF-1$\alpha$ expression on treatment with NO-sulindac. The greatest reduction in HIF-1$\alpha$ was at the 0.2% oxygen level compared to the control treated cells. Figure 4b illustrates an increase in the level of HIF-1$\alpha$ protein stabilization following sulindac treatment of the PC-3 cells at the 4 different oxygen levels. Immunocytochemistry (Fig. 5a) also demonstrated that under hypoxia there was an increase in HIF-1$\alpha$ translocation to the nucleoplasm, but not the nucleolus. NO-sulindac reduced nuclear translocation of HIF-1$\alpha$ in hypoxic PC-3 cells.

**NO-sulindac downregulates activation of the hypoxia response element promoter region**

PC-3 cells transiently transfected with a luciferase gene under the control of the hypoxia response element (HRE) were employed to evaluate the consequences of NO-sulindac treatment on HIF-1$\alpha$ transcriptional activity. Figure 5b illustrates a 5.3-fold increase in HRE promoter activation following incubation at 0.2% oxygen compared to 21% oxygen. Treatment with 25 $\mu$M NO-

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**Figure 2** – Cytotoxic effect of NO-sulindac under varying oxygen conditions. A determination of the cytotoxic effect of NO-sulindac and its derivatives on PC-3 cells was made using PI uptake and flow cytometric analysis. (a) The effect of differing doses of NO-sulindac and sulindac on normoxic PC-3 cells, relative to DMSO vehicle control treated cells. (b) Bar graph showing the differing cytotoxic effects and nitrite concentrations of 25–50 $\mu$M: sulindac, combined sulindac + SNP (established NO-donor), NCX 221 (denitrated analogue of NO-sulindac) and NO-sulindac on PC-3 cells after 48 hr incubation at normoxia. Nitrite concentrations of PC-3 cell medium, measured using the Griess assay, following each treatment are documented above the corresponding graph bar. Differences in cell survival between NO-sulindac and NCX 221 treated cells are shown as, $^{a}p < 0.005$. (c) The effect of NO-sulindac or (d) sulindac on PC-3 cells incubated for 48 hr under differing oxygen conditions. Survival expressed relative to PC-3 cells treated with the DMSO vehicle control at 21% oxygen. Differences from control at the same oxygen level are shown as, $^{a}p < 0.05$, $^{b}p < 0.005$, $^{c}p < 0.0005$, Mann-Whitney U test. There was no significant difference between the relative survival of sulindac treated PC-3 cells and control treated cells at any oxygen level.
sulindac at 0.2% oxygen resulted in a 31.2% relative reduction in HRE promoter activation compared to the DMSO control (p = 0.028, Mann-Whitney U test). Whereas, treatment with 25 μM sulindac resulted in a 5.2% increase in HRE promoter activation.

NO-sulindac reduces the mRNA expression of selected hypoxia associated genes

Table I details the fold-change in selected hypoxia associated gene expression following PC-3 cell incubation at 0.2% oxygen. LOX and CAIX were upregulated 2.3- and 6.1-fold respectively following incubation of PC-3 cells at 0.2% oxygen compared to 21% oxygen incubation. HIF-1α mRNA expression by hypoxic PC-3 cells was downregulated to 0.6-fold of the level found under normoxia.

LOX and CAIX mRNA expression showed the same pattern found with the HIF-1α protein and HRE promoter reporter construct following the treatment of PC-3 cells with sulindac or NO-sulindac (Table I). PC-3 cell treatment with 25 μM or 50 μM NO-sulindac under 0.2% oxygen conditions resulted in a reduction in LOX and CAIX mRNA. There was also a reduction in LOX mRNA expression following sulindac treatment; however, this was not as great a reduction as with NO-sulindac.

CAIX gene expression was reduced by the 50 μM sulindac used but slightly increased by 25 μM sulindac. HIF-1α mRNA expression was reduced by 50 μM NO-sulindac and both doses of sulindac used. As such, HIF-1α did not show a well-defined alteration in the pattern of mRNA expression and did not exhibit the same downregulation observed in HIF-1α at the protein level.

Reduction of HIF-1α expression is partially responsible for the hypoxia induced resistance of PC-3 cells to NO-sulindac

After incubating for 48 hr under both normoxia and hypoxia, the relative survival of control treated PC-3 cells transfected with HIF-1α RNAi was significantly lower than cells transfected with the negative control RNAi (Fig. 5c). NO-sulindac treatment of HIF-1α RNAi transfected, normoxic PC-3 cells did not result in an increase in cell death over negative control transfected cells. However, under hypoxic conditions there was a significant reduc-
tion in HIF-1α RNAi transfected PC-3 cell survival following NO-sulindac treatment compared to negative control transfected cells. Under these conditions, cell survival was reduced to the same level as following 25 μM NO-sulindac treatment under normoxic conditions (p = 0.2, Mann-Whitney U test), i.e., hypoxia induced chemoresistance was overcome.

**Inhibition of HIF-1α accumulation by NO-sulindac in PC-3 cells is independent of proteasomal degradation**

Under normoxia HIF-1α is normally degraded by ubiquitination and subsequent proteolysis within the proteasome. The hypothesis that the HIF-1α degradation induced by NO-sulindac was due to promotion of the proteasome pathway was tested by pre-treating...
PC-3 cells with the proteasome inhibitor MG132 whilst co-administering NO-sulindac and incubating under hypoxic conditions. Pre-treatment of normoxic PC-3 cells with MG132 upregulated HIF-1α expression (Fig. 6a). However, the inhibition by NO-sulindac of HIF-1α accumulation in response to hypoxia was not blocked by MG132. As such, the inhibition of HIF-1α by NO-sulindac was not due to proteasomal degradation.

**NO-sulindac inhibits HIF-1α via the PI3K/Akt pathway under hypoxia**

Treatment of hypoxic PC-3 cells with the PI3K inhibitor LY294002 under hypoxic conditions for 48 hr. Western blot analysis revealed a dose dependent, blocked by MG132. As such, the inhibition of HIF-1α by NO-sulindac was not due to proteasomal degradation.

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**Table 1 – Real-time PCR quantification of the fold-change of hypoxia-associated gene expression in (i) untreated PC-3 cells incubated at 0.2% oxygen (relative to gene expression at 21% oxygen) and (ii) hypoxic PC-3 cells treated with sulindac or NO-sulindac (relative to gene expression in hypoxic/DMSO vehicle control treated cells).** Results expressed as fold change ± SD.

<table>
<thead>
<tr>
<th>Oxygen treatment</th>
<th>HIF-1α</th>
<th>CAIX</th>
<th>LOX</th>
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<td>(i) Oxygen treatment</td>
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<td>0.2% oxygen</td>
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<td>(ii) Drug treatment</td>
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SD, standard deviation.

**Discussion**

The data presented earlier illustrates novel tumouricidal and anti-invasion properties of NO-sulindac in a hypoxic microenvironment. Under low oxygen conditions, NO-sulindac directly inhibited the hypoxia response of hormone-resistant PC-3 prostate cancer cells in vitro via Akt signalling. NO-sulindac was cytotoxic in a dose- and time-of-exposure-dependent fashion.

Previous work from our laboratory determined that NO-aspirin and NO-ibuprofen inhibited proliferation and induced apoptosis in a dose-dependent fashion in PC-3, LNCaP and prostatic stromal cells. NO-aspirin and NO-ibuprofen were both more effective at inhibiting proliferation and inducing apoptosis than conventional

![Figure 6](image-url)

**Figure 6 –** (a) The role of the proteasome in degradation of HIF-1α. PC-3 cells were treated for 4 hr under normoxic or hypoxic conditions with proteasome inhibitor MG132 (10 μM) and for 48 hr with 50 μM NO-sulindac before immunoblotting. Proteasome inhibition with MG132 caused an accumulation of HIF-1α. NO-sulindac treatment resulted in a reduction in nuclear HIF-1α protein; however, the co-administration of MG132 with NO-sulindac did not alter HIF-1α levels. (b, c) NO-sulindac induced inhibition of HIF-1α via the Akt pathway under hypoxia. PC-3 cells were incubated with the PI3K inhibitor LY294002 under hypoxic conditions for 48 hr. Western blot analysis revealed a dose dependent, concurrent reduction in p-Akt (65 kDa) and HIF-1α following LY294002 treatment. (c) Before immunoblotting for p-Akt and HIF-1α, PC-3 cells were pretreated with 0.05% DMSO or 25 μM NO-sulindac followed by hypoxic incubation for 48 hr. HIF-1α and p-Akt protein levels were both reduced following NO-sulindac treatment. (d) PC-3 cells cotransfected with pGL2-Tk-HRE and myr-Akt (or empty vector pUSEamp (ev)) plasmids were treated with 0.05% DMSO or 5–25 μM NO-sulindac for 48 hr under hypoxic conditions. Transfection of PC-3 cells with myr-Akt prevented the reduction in activation of the HRE following NO-sulindac treatment, in a dose-dependent fashion. Data represents the fold increase in luciferase expression relative to that of the empty vector control in the presence of DMSO. Increased p-Akt protein levels following transfection were determined by immunoblot.
Aspirin, ibuprofen or the NO-donor SNP. Previous studies using NO-sulindac demonstrated a cytotoxic and antiproliferative effect on normoxic LNCaP and PC-3 cells.35,36 In these previous studies by Huguenin et al.,37 NO-sulindac was also found to alter the cell cycle, induce mitotic arrest and display pro-apoptotic activity in malignant and benign cell lines. The results of the normoxia experiments in the present study concur with these previous results showing a reduction in PC-3 cell viability in a dose- and time-dependent manner following treatment with NO-sulindac as well as a comparable rate of apoptosis.

Recent studies, from several research groups, have established that the cytotoxic activity of NO-aspirin has little or nothing to do with the NO-releasing or aspirin groups and that the linker/spacer is biologically active.37–40 As such, we assessed the cytotoxic effect and NO-releasing properties of the various components of the NO-sulindac compound. Previous studies have suggested that the anti-tumour effect of NO-aspirin involves a quinone methide (formed from the hydroxybenzyl linker) but neither nitric oxide nor aspirin.38,40 However, this expectation does not extend to other linker structures40; the spacer found in NO-sulindac is a butyloxy linker which does not form a quinone. In vivo and in vitro, unlike NO-aspirin, NO-sulindac is predicted to function as a NO-donor and deliver ‘NO’ bioactivity (personal communication, Professor Gregory Thatcher (University of Illinois, Chicago)). However, such effects are likely to be reduced in cell culture as nitrates require metabolic bioactivation to liberate NO+ bioactivity, which is often poorly replicated in vitro.40 The low levels of nitrite released by NO-sulindac concur with these predictions, it may be that the NO+ release would be higher in vivo. In the present study, despite releasing a much higher concentration of NO+, the combination of SNP + sulindac was significantly less cytotoxic than NO-sulindac. The denitrogated analogue of NO-sulindac (i.e., sulindac + spacer) had an intermediate cytotoxic effect, greater than DMSO vehicle control but lower than NO-sulindac. Taken together, these findings suggest that the spacer of NO-sulindac is not inert and that the NO-releasing group provided some of the cytotoxic effect of NO-sulindac (unlike NO-aspirin where the NO-releasing moiety does not appear to be needed40). It remains to be determined if the sulindac moiety is required in combination with both the linker and NO-releasing group to achieve the same biological activity.

It is well established that solid human tumours exist under hypoxic conditions and that this is the case within a nidus of prostate cancer.18,19 Hypoxia is known to increase the resistance of tumours to chemotherapeutic drugs.20 As such, it was important to establish the cytotoxic effects of NO-sulindac under hypoxia. Oxygen levels of less than 1% O2 (pO2 ~10 mmHg) characterize tissues which have compromised blood flow such as prostate cancer which have been demonstrated to have an average median pO2 of 2.4 mmHg.18,41 As such, 0.2% oxygen was used in the experiments of the present study to mimic the median pO2 in vivo. This choice was ratified by the consistent upregulation of HIF-1α, as a surrogate for induction of the hypoxia response, at 0.2% oxygen but neither 5 nor 10% oxygen. The results from the current study showed that as the oxygen levels fell, the relative survival of PC-3 cells decreased to 75% viability at 10% oxygen, 68% at 5% oxygen and 62% at 0.2% oxygen. Although atmospheric oxygen concentration is 21%, the level of oxygen in tissues in vivo is significantly lower (physiological hypoxia) with a mean tissue oxygen concentration ~3%.42 Thus, incubation of PC-3 cells at 5% and 10% oxygen was likely to represent physiological rather than pathological oxygen levels. These in vitro findings suggest that such physiological hypoxia resulted in PC-3 cell death perhaps reflects the difficulty in attempting physiological studies in tissue culture.

Treatment of PC-3 cells with 25 μM or 50 μM of NO-sulindac at the 4 different oxygen levels resulted in a significant increase in cell death relative to the control. However, a degree of chemoresistance was introduced by hypoxia. At both 25 μM and 50 μM doses of NO-sulindac, cell killing was greatest at 21% oxygen. As the oxygen level was reduced there was an increase in the relative PC-3 cell survival. However, at both doses of NO-sulindac (but not sulindac) used there was still a significant reduction in survival relative to the PC-3 cells treated with the vehicle control. Other than a doubling of the proportion of cells undergoing apoptosis relative to hypoxia (but only from 0.8 to 1.7%), there were no major cell cycle differences between hypoxic and normoxic PC-3 cells. As such, a slowing of the cell cycle in hypoxic cells was not responsible for the reduced effect of NO-sulindac on hypoxic PC-3 cells. A previous study demonstrated that hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer was inhibited by silencing of HIF-1α mRNA.43 Similarly, in the present study, knockdown of HIF-1α mRNA combined with NO-sulindac treatment reversed the chemoresistance induced by hypoxia, suggesting that HIF-1α expression under hypoxia may be responsible for hypoxia induced chemoresistance. However, despite chemoresistance these results demonstrate that overall NO-sulindac has significant cytotoxic effects under hypoxia.

Hypoxia is associated with increased invasive and metastatic potential. Previous studies have shown that the increased metastasis induced by hypoxia was inhibited in murine melanoma cells by co-incubation with low concentrations of the NO-mimetic drugs glyceryl trinitrate and diethylentriamine NO+ adduct (DETA/NO).44 The significant reduction in PC-3 cell invasion caused by NO-sulindac under normoxia and hypoxia suggests that NO-sulindac may also act to prevent invasion and metastasis of prostate cancer cells in vivo.

The immunoblot, immunocytochemical and HRE promoter reporter construct data confirm that the HIF-1α protein and its transcriptional activity were inhibited by NO-sulindac under normoxic and hypoxic conditions by up to 31%. Quantitative real-time PCR was performed to assess the transcriptional activity of the hypoxic PC-3 cells had on a number of hypoxia-associated genes. As expected, mRNA of the hypoxia-associated genes: CAIX and HIF-1α was downregulated in NO-sulindac treated cells. A previous study demonstrated that hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer was inhibited by silencing of HIF-1α mRNA.43 Similarly, in the present study, knockdown of HIF-1α mRNA combined with NO-sulindac treatment reversed the chemoresistance induced by hypoxia, suggesting that HIF-1α expression under hypoxia may be responsible for hypoxia induced chemoresistance. However, despite chemoresistance these results demonstrate that overall NO-sulindac has significant cytotoxic effects under hypoxia.

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Taken together, these findings suggest that the spacer of NO-sulindac is not inert and that the NO-releasing group provided some of the cytotoxic effect of NO-sulindac (unlike NO-aspirin where the NO-releasing moiety does not appear to be needed40). It remains to be determined if the sulindac moiety is required in combination with both the linker and NO-releasing group to achieve the same biological activity.
vent the general reduction in translation rates that occur under hypoxic conditions.46

A number of previous studies have demonstrated the link between the PI3K-Akt-mTOR signal transduction pathway and control of HIF-1α expression under hypoxia.47 As such, the effect of NO-sulindac on this axis was investigated further. The present study demonstrated that HIF-1α nuclear protein levels were reduced in parallel with phosphorylation of Akt by both PI3K inhibitor (LY294002) and NO-sulindac. Experiments using PC-3 cells co-transfected with the HRE promoter reporter and constitutively expressing Akt plasmids linked Akt phosphorylation to HIF-1α production by PC-3 cells under hypoxia, suggesting that this is one mechanism by which NO-sulindac functions. Other HIF-1α inhibitors, such as YC-1, also regulate HIF-1α transactivation via the PI3K-Akt-mTOR signal transduction pathway.48 Clearly, there may be other mechanisms at play in the regulation of HIF-1α by NO-sulindac.

A notable finding of this study was that while the relative amount of cell death caused by NO-sulindac fell with diminishing oxygen levels, HIF-1α protein accumulation was also reduced. This raises the question of why under hypoxia, cell death due to NO-sulindac was not greater, as NO-sulindac caused a reduction in hypoxia-associated proteins, potentially preventing adaption of the hypoxic cells to the harsh microenvironment. Intuitively, HIF-1α expression is expected to be inversely proportional to the level of cytotoxicity observed under hypoxic conditions. Furthermore, results of RNAi experiments showed that, under hypoxia, knock-down of HIF-1α alone caused a greater reduction in PC-3 cell survival than NO-sulindac. However, HIF-1α protein was reduced to a lesser degree (~20–30% reduction) by 25–50 μM NO-sulindac than by RNAi, suggesting that although the reduction in HIF-1α by NO-sulindac may be partly responsible for its cell death induction it was not the sole reason. Additionally, similar studies evaluating the HIF-1α inhibitor YC-1 showed that the inhibition of HIF-1α accumulation by YC-1 under hypoxia was not attributable to cell death.49

This study determined for the first time that NO-sulindac is significantly more active than sulindac as a cytotoxic, anti-invasion agent against the PC-3 hormone-insensitive prostate cancer cell line under hypoxia. NO-sulindac directly inhibits the HIF-1α induced hypoxia response via the inhibition of Akt transactivation. The ability of NO-sulindac to inhibit tumour adaptation to hypoxia potentially has far-reaching implications and is of considerable relevance to the future management of hormone-insensitive prostate cancer with the same cellular characteristics as PC-3 cells. In vivo confirmation of the results from this study is now required. However, combination of pre-clinical evidence data presented here and elsewhere, epidemiological evidence of the role of NSAIDs in prostate cancer prevention and gastroprotective benefit of NO-NSAIDs suggest that NO-sulindac is potentially a useful agent for the future treatment of prostate cancer.

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References


