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**DNA strand breaks and hypoxia response inhibition mediate the
radiosensitisation effect of nitric oxide donors on prostate cancer under varying
oxygen conditions**

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Abbreviations: NO, nitric oxide; NO-NSAIDs, nitric oxide donating non-steroidal
anti-inflammatory drugs; Gy, Gray; PE, plating efficiency; SF, surviving fraction;
SER, sensitiser enhancement ratio; OER, oxygen enhancement ratios; DSB, DNA
double strand break; HIF-1 α , hypoxia inducible factor-1 α .

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Abstract

Prostate cancer cells can exist in a hypoxic microenvironment, causing radioresistance. Nitric oxide (NO) is a radiosensitiser of mammalian cells. NO-NSAIDs are a potential means of delivering NO to prostate cancer cells. This study aimed to determine the effect and mechanism of action of NO-sulindac and radiation, on prostate cancer cells and stroma, under normoxia (21% oxygen) and chronic hypoxia (0.2% oxygen). Using clonogenic assays, at a surviving fraction of 10% the sensitisation enhancement ratios of radiation plus NO-sulindac over radiation alone on PC-3 cells were 1.22 and 1.42 under normoxia and hypoxia, respectively. 3D culture of PC-3 cells revealed significantly reduced sphere diameter in irradiated spheres treated with NO-sulindac. Neither NO-sulindac nor sulindac radiosensitised prostate stromal cells under normoxia or hypoxia. HIF-1 α protein levels were reduced by NO-sulindac exposure and radiation at 21% and 0.2% oxygen. Alkaline Comet assay analysis suggested an increased rate of single strand DNA breaks and slower repair of these lesions in PC-3 cells treated with NO-sulindac prior to irradiation. There was a higher level of γ -H2AX production and hence double strand DNA breaks following irradiation of NO-sulindac treated PC-3 cells. At all radiation doses and oxygen levels tested, treatment of 2D and 3D cultures of PC-3 cells with NO-sulindac prior to irradiation radiosensitised PC-3, with minimal effect on stromal cells. Hypoxia response inhibition and increased DNA double strand breaks are potential mechanisms of action. Neoadjuvant and concurrent use of NO-NSAIDs have the potential to improve radiotherapy treatment of prostate cancer under normoxia and hypoxia.

Key words: hypoxia; prostate cancer; nitric oxide donors; radiation;
radiosensitisation.

1. Introduction

Both localised and locally advanced prostate cancers are commonly treated by radiotherapeutic means; either external beam radiotherapy or brachytherapy. However better strategies are required to improve therapeutic gain of radiotherapy. This is especially the case in locally advanced prostate cancer, where the effectiveness of radiotherapy is limited by the bulk of disease being treated and dose delivery being limited by the normal-tissue tolerance of radiation. Additionally, hypoxia is a factor at play in the microenvironment of most solid tumours, including prostate cancer [1]. Amongst other effects, hypoxia causes radioresistance in prostate cancer hindering the optimal treatment of these tumours by radiotherapy [1].

Nitric oxide donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) are novel pharmaceutical agents which were developed to allow NSAIDs to be better tolerated due to their associated gastro-protection. The nitric oxide (NO) group of NO-NSAIDs confers a protective effect on 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 [2]. NO-NSAIDs combine the anti-proliferative effects of NSAIDs with the gastric protection and potential tumouricidal effects of NO [3]. We and others have demonstrated that NO-NSAIDs prevent the development of malignancy and are powerful agents against established cancer deposits *in vitro* and *in vivo* [4-6]. Recently we have determined the function of NO-sulindac under hypoxic conditions and shown that this agent inhibits the hypoxia response in the PC-3 prostate cancer cell line via the Akt pathway [7]. Independently, NO and NSAIDs have both been shown to behave as radiosensitising agents [8, 9]. Based on this evidence we hypothesised that NO-sulindac would act as a radiosensitiser in prostate cancer under normoxic and hypoxic

conditions. The results confirmed our hypothesis and mechanistic studies revealed that inhibition of the hypoxia response and DNA strand break was responsible for this phenomenon.

2. Materials and Methods

2.1. Cell culture and reagents

Human prostatic stroma cells were grown as previously described from the transurethral prostatectomy chippings of men with benign prostatic hyperplasia [10]. National Research Ethics Service approval (REC number 02/5/063) and informed consent was obtained for tissue donation. PC-3 cells (obtained from the European Collection of Cell Cultures, Salisbury, UK) were cultured in RPMI-1640 with 10% FCS (all from Invitrogen, Paisley, UK). PC-3 cells were seeded into 25cm³ flasks at 2x10⁶ cells/flask and stromal cells at 1x10⁶ cells/flask for all experiments. NO-sulindac (NCX 1102) and sulindac were donated by NicOx (Sophia Antipolis, France) and prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Gillingham, UK) with final DMSO concentrations of 0.05%. Chronic hypoxia was induced by incubating PC-3 cells for 48 hours within a humidified hypoxia incubator at 0.2% oxygen using a PROOX 110 oxygen controller (BioSpherix Ltd, Redfield, NY).

2.2. Radiation treatments

A custom made Perspex jig was produced by the Edinburgh Cancer Centre Workshop (Western General Hospital, Edinburgh, UK) to allow standardised irradiation of cells in 25cm³ flasks. Following cell treatment as per experimental protocol, cells were irradiated using a Varian 2100C/D linear accelerator (Palo Alto, CA) at a dose rate of 0.527Gy/min at 15mV photons to a dose of 2-8Gy. Thermoluminescent dosimeters measurements were performed, and demonstrated that the radiation doses received by cells was homogeneous across the base of all the flasks.

2.3. Clonogenic assay

To assess reproductive ability of cells following irradiation a clonogenic assay was employed as described previously [11]. PC-3 cells were incubated for 14-21 days and primary prostate stromal cells for 10-14 days until cell colonies were visible to the naked eye, but not coalescing. Plating efficiency and surviving fraction (SF) were calculated in the standard fashion [11] using the result of at least three independent experiments, each experiment including 3 to 6 dishes. Radiation survival curves were plotted after normalizing for the cytotoxicity induced by drug treatment alone. Linear-quadratic curve equations were used to fit the SF curves shown in the figures using KaleidaGraph 4.0 (Synergy Software, PA). Radiation sensitiser enhancement ratio (SER) for NO-sulindac or sulindac treatment was calculated using the radiation dose required to reduce clonogenic survival to 10% (D_{10} , given in Gy). The SERs reported are the D_{10} values for control curves divided by the values for treated curves in each instance [12]. Oxygen enhancement ratios (OER) were calculated by dividing the D_{10} value under hypoxia by the D_{10} value under normoxic conditions.

2.4. 3D cell culture

PC-3 cells were treated as above before harvesting and counting for 3D culture. Single cell solutions were mixed at a ratio of 1:2 with a reconstituted basement membrane of growth factor reduced Matrigel (BD Biosciences, Oxford, UK) to a final density of 100,000 cells/ml. The mix was layered thinly on plastic and left to gel for 1 hour at 37°C. The gels were then covered with standard PC-3 medium for 5 days. Medium was replaced at 3 days. For acinar structure size calculations, diameters of 50

distinct structures were measured using a tissue culture microscope and a graticule at 200x magnification.

2.5. *Single-cell gel electrophoresis (alkaline Comet assay)*

DNA damage was measured using the alkaline Comet assay and expressed as percentage of DNA in the tail of the comet (% tail DNA) as described previously [13]. Results were expressed as the mean and the standard deviation of at least three independent samples.

2.6. *γ -H2AX assay*

Radiation induced DNA double strand break (DSB) formation was determined by the γ -H2AX flow cytometry assay, with relative DSB levels being determined by γ -H2AX fluorescence intensities as described previously [14]. Results were expressed as the mean and the standard deviation of three independent samples.

2.7. *HIF-1 α western blot*

Western blots for hypoxia inducible factor (HIF)-1 α were performed on nuclear protein extracts as described previously [7, 15].

2.8. *Statistical analysis*

Each experiment was repeated at least three times. All values are expressed as means $\pm$ standard deviation (SD). Student's t-test was used for true pairwise comparisons and ANOVA with post-hoc Dunnett's multiple comparison test for many to one treatment to vehicle comparisons. Calculations were performed using SPSS 13.0 (Chicago, IL); P<0.05 was considered significant.

3. Results

3.1. *Oxygen enhanced the effect of radiation on PC-3 cells and primary prostate stromal cells*

PC-3 and primary prostate stromal cells were incubated under normoxia or hypoxia for 48 hours in the presence of 0.05% DMSO (vehicle control for NO-sulindac experiments). Following irradiation at 2, 4, 6 or 8Gy of ionizing radiation, cells were harvested and clonogenic assays conducted. The hypoxia survival curves for irradiated PC-3 (Fig. 1A) and stromal cells (Fig. 2A) both separate from the normoxic survival curves indicating a degree of radioresistance introduced by hypoxia. However, the only significant difference between the curves was for PC-3 cells at 8Gy radiation. The OER for PC-3 cells was 1.13 and for stromal cells 1.19 (see table 1 for D_{10} values).

3.2. *NO-sulindac radiosensitises PC-3 prostate cancer cells under both normoxia and hypoxia*

PC-3 cells were treated with 25 μ M NO-sulindac, 25 μ M sulindac or vehicle control for 48 hours under either 21% or 0.2% oxygen. Without irradiation, the relative survival of PC-3 cells (relative to control-treated cells) treated with 25 μ M NO-sulindac was 0.27 at 21% oxygen and 0.34 at 0.2% oxygen and following 25 μ M sulindac treatment 1.12 at 21% oxygen and 0.62 at 0.2% oxygen. Following drug treatment $\pm$ hypoxia, cells were irradiated with 2-8Gy of ionizing radiation, prior to undertaking clonogenic assays. From these experiments it was possible to determine the D_{10} values of individual drug treatments (table 1). These results reveal that whilst greater radiation doses were required to reduce clonogenic survival to 10% under hypoxic conditions, NO-sulindac treatment reduced the radiation dose required to

reach D_{10} . The radiosensitising effects of NO-sulindac on PC-3 cells over the dose range 2-8Gy, under both normoxia and hypoxia, are shown in Fig. 1B and 1C. After normalising drug survival curves for the effects of NO-sulindac/sulindac/DMSO at 0Gy there remained a significant separation of the radiation/NO-sulindac curves compared with radiation/DMSO control for both normoxia and hypoxia ($P < 0.04$, ANOVA). The SER values were 1.22 and 1.42 for NO-sulindac under normoxia and hypoxia respectively. The SER for sulindac treatment of PC-3 cells were 0.93 and 1.13, under normoxia and hypoxia, respectively.

3.3. NO-sulindac does not radiosensitise primary prostate stromal cells at 2-6Gy

The same experimental set-up was repeated with primary prostate stromal cells (Fig. 2). NO-sulindac and sulindac did not radiosensitise prostate stromal cells under normoxia or hypoxia following irradiation at 2, 4 or 6Gy ($P > 0.05$, ANOVA). However, at 8Gy under normoxia ($P = 0.01$, ANOVA), but not hypoxia ($P = 0.06$, ANOVA), there was significant separation of the curves for NO-sulindac and control-treated cells.

Without irradiation, the relative survival of stromal cells (relative to control-treated cells) treated with 25 μ M NO-sulindac was 0.56 at 21% oxygen and 0.37 at 0.2% oxygen and following 25 μ M sulindac treatment 0.97 at 21% oxygen and 1.1 at 0.2% oxygen. The SER values of stromal cells were 0.96 and 1.02 for NO-sulindac under normoxia and hypoxia respectively. The SER for sulindac treatment of stromal cells were 0.93 and 1.04, under normoxia and hypoxia respectively. Thus, NO-sulindac had a non-interactive combined effect with radiation and it is not a radiosensitiser of stromal cells.

3.4. Reduction in PC-3 3D sphere diameter was greatest following NO-sulindac treatment and irradiation of hypoxic PC-3 cells

Cultures of glandular epithelial cells in reconstituted basement membrane (3D cultures) are an established method of examining tumourigenic potential [16, 17]. In these cultures, epithelial cells undergo subsequent steps of proliferation, polarisation and lumen formation through apoptosis of cells inside the acinar structure formed [18]. Exposure to 4Gy of radiation at 21% oxygen was sufficient to decrease the size of acinar structures by approximately 2-fold (Fig. 3A and 3C). While unexposed PC-3 cells proliferated and created a mature lumen within 5 days (Fig. 3C), irradiated cells proliferated less, in agreement with the colony formation assays (Fig. 3A-D). Hypoxia on its own had a similar but smaller effect on proliferation, ~1.3 fold reduction in sphere size (Fig. 3B and 3D). NO-sulindac treatment of PC-3 cells exposed to both hypoxia and radiotherapy resulted in even smaller structures than DMSO or sulindac treatment with radiotherapy (Fig. 3B and 3D; $P < 0.01$, ANOVA). Under hypoxic conditions exposure of PC-3 cells to NO-sulindac prior to irradiation resulted in a significant reduction in sphere diameter compared with control treatments (Fig. 3B and 3D). All treatments resulted in failure of the cells to generate acinar structures with mature lumens (data not shown). This evidence strongly suggests that the main effects observed are anti-proliferative and therefore will result in lower tumourigenicity *in vivo* [16, 19, 20]. Furthermore, these 3D culture results agree with the results from 2D culture.

3.5. HIF-1 α nuclear protein levels were reduced by NO-sulindac and radiation under normoxia and hypoxia

The level of HIF-1 α present in the nuclear fraction of PC-3 cells was assessed using western blotting. As has been demonstrated previously, HIF-1 α nuclear protein was present under normoxic conditions in PC-3 cells (Fig. 4A), in contrast to many other cell lines [21]. As expected HIF-1 α levels were elevated by incubation of PC-3 cells under hypoxic conditions for 48 hours (Fig. 4A). However, under both normoxia and hypoxia there was a serial reduction in HIF-1 α levels following irradiation at 2Gy at 8, 24 and 48 hours (Fig. 4A). As we have shown previously, treatment of PC-3 cells with 25 μ M NO-sulindac under both normoxia and hypoxia reduces the levels of HIF-1 α [7]. Furthermore, HIF-1 α levels were undetectable on western blotting following NO-sulindac and radiation treatment at 8, 24 or 48 hours post-irradiation (Fig. 4B).

The effect of increasing radiation dose combined with NO-sulindac treatment on HIF-1 α expression was also assessed. Fig. 4C and 4D shows the effect of an increasing radiation dose (2 to 8Gy) on PC-3 cells under normoxia (Fig. 4C) or hypoxia (Fig. 4D) with each additive. Under both normoxia and hypoxia, increasing the radiation dose from 2 to 8Gy resulted in a reduction in HIF-1 α expression (as seen in the nuclear protein of DMSO treated cells; Fig. 4C and 4D). Treatment of PC-3 cells with 25 μ M NO-sulindac prior to irradiation resulted in a reduction in HIF-1 α nuclear protein levels under both normoxia and hypoxia and at both 2 and 8Gy. Furthermore, there was a reduction in HIF-1 α protein with 25 μ M sulindac under normoxia at 2 and 8Gy, this effect of sulindac was not noticeable under any other conditions.

3.6. NO-sulindac stimulates the formation of radiation-induced single strand DNA breaks (SSB) and double strand breaks whilst reducing DNA repair in PC-3 cells

1 PC-3 cells were irradiated at 2 and 4Gy under normoxia with each additive,
2 following which an alkaline Comet assay was performed. Fig. 5A demonstrates that at
3
4 4Gy, 48 hours of NO-sulindac treatment resulted in a significant increase in SSB
5 formation compared with DMSO vehicle control or sulindac. There was no significant
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7 difference between the different treatments in SSB formation found at 0 or 2Gy.
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11 As a surrogate for DSB formation, phosphorylation of the histone H2AX to γ -
12 H2AX was determined using immunohistochemical detection. At 4Gy, under
13
14 normoxia there were significantly greater levels of DSBs in PC-3 cells 1 and 2 hours
15 following irradiation in the presence of NO-sulindac than sulindac (Fig. 5B).
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19 Previous studies have demonstrated longer repair times for DSB (measured as
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21 γ -H2AX foci) for cells irradiated in the presence of nitric oxide [22]. To assess the
22 effect of NO-sulindac on the DNA strand break repair, PC-3 cells were treated with
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24 NO-sulindac, sulindac or DMSO for 48 hours. The cells were then irradiated (8Gy)
25 and incubated for up to 60 minutes to assess the extent of repair using the Comet
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27 assay. Fig. 5C illustrates the SSB repair process over a 60 minute period. PC-3 cell
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29 treatment with NO-sulindac significantly slows the extent of SSB repair compared
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31 with sulindac or DMSO treatment at 15, 30 and 45 minutes. PC-3 cell treatment with
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33 sulindac did not affect the rate or extent of SSB repair compared with the control
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35 treated cells. Additionally, longer repair times for DSBs were suggested by the lack of
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37 significant decrease in the extent of γ -H2AX formation 1 and 2 hours following
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39 irradiation of NO-sulindac treated cells compared with the significant decrease noted
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41 in the irradiated control cells.
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4. Discussion

The findings of this study demonstrate that NO-donors have the potential to act as radiosensitisers in prostate cancer. This work complements and expands our previous studies which demonstrated the cytotoxic effects of NO-NSAIDs on prostate cancer cells under normoxia and hypoxia [6, 7]. The use of both 2D and 3D models showed that NO-sulindac radiosensitised the PC-3 hormone insensitive prostate cancer cell line, not only under normoxia but particularly under chronic hypoxia. Conversely, prostate stromal cells were not radiosensitised by either NO-sulindac or sulindac. The reduction in nuclear HIF-1 α expression by irradiated PC-3 cells pre-treated with NO-sulindac suggests that dampening of the hypoxia response may have a role to play. Finally, a radiobiological mechanism for the radiosensitisation caused by NO-sulindac was determined to be an increase in the extent of single and double strand DNA breaks together with reduced speed of repair of these breaks. Taken together these findings suggest that NO-NSAIDs have potential as neoadjuvant and concurrent treatments for men undergoing radiotherapy for prostate cancer.

We recognise the lack of an *in vivo* component of this study. However, in an attempt to mimic the *in vivo* situation, an *in vitro* 3D model has been utilised, which has previously been shown to behave similarly to a mouse xenograft model [20]. It is not possible to mimic the chaotic, oxygenation of a solid tumour deposit in the *in vitro* setting. As a result, the OERs obtained in this study were lower than expected. For most cells the OER following exposure to x-rays is ~3.0 [23], in the data presented here the OERs were 1.1 for PC-3 cells and 1.2 for stromal cells. However, previous detailed *in vitro* studies have demonstrated that the OER following exposure to acute hypoxia (3 hours) was ≥ 2.0 ; whereas, following chronic hypoxia (24 to 72 hours), the OER was reduced to 1.1 in the same cell lines [24]. The reduced OER under chronic

hypoxia was most common in p53 mutant cell lines such as PC-3, helping to explain the OERs in the study presented here [24, 25]. Furthermore, the upregulation of HIF-1 α confirmed that hypoxic conditions were achieved.

The results presented here show that NO-sulindac provides a radiosensitising effect, which was maximal under hypoxia. This is a convenient finding as hypoxia is a tumour microenvironmental condition contributing to human prostate cancer radioresistance [1]. NO-sulindac was found to have a SER of 1.22 under normoxia and 1.45 under hypoxic conditions in PC-3 cells. Dose escalation trials in prostate cancer external beam radiotherapy, such as RT01, have shown improved effectiveness of 74-79.2Gy dose range at 1.8-2Gy per fraction with respect to clinical outcomes compared with lower radiation dose regimens [26]. Crucially, at 2Gy, NO-sulindac resulted in a significant radiosensitising effect over control treated cells under both normoxia (up to 2.2 fold reduction in cell survival) and hypoxia (1.9 fold reduction in survival). These differences in survival would give a major therapeutic advantage in a multiple fraction treatment regimen. The radiosensitising effects of oxygen and misonidazole are reduced at low radiation doses [27, 28]. However, studies by Wardman and co-workers demonstrated that NO (1% v/v, ~19 μ M) was effective as a radiosensitiser at low, clinically relevant radiation doses (2Gy) in anoxic cells [22]. The results of the present study, which also showed radiosensitisation at similar low doses, concur with the findings of Wardman *et al.*

In addition to the radiosensitising effect of NO-sulindac on prostate cancer cells, the studies presented here also assessed the effect this compound had on prostate stromal cells. Unlike prostate epithelial cells, treatment with NO-sulindac did not sensitise stromal cells to irradiation under either normoxia or hypoxia at the clinically relevant doses (1.8-2.0Gy/fraction). The difference in radiosensitising

ability of NO-sulindac between PC-3 epithelial cells and primary stromal cells may simply be a function of the increased resistance of stromal cells to radiation or NO-sulindac (relative survival of stromal cells following treatment with 25 μ M NO-sulindac, 0.56 at 21% oxygen and 0.37 at 0.2% oxygen, compared with 0.27 and 0.34 respectively for PC-3 cells). However, an alternative message may be taken from the results of stromal cell irradiation with NO-sulindac. There is disagreement between studies, but there is evidence showing that skin fibroblasts act as surrogates for normal tissue in a radiobiological sense, and predict the severity of normal tissue damage [29]. A case report in prostate cancer showed that skin fibroblast radiosensitivity predicted a patient developing rectal toxicity following irradiation [30]. As prostate stromal cells have a predominance of fibroblasts it can be speculated that the effect of irradiation on prostate stromal cells will also predict the potential effects on normal tissues such as the bladder, rectum or skin [31]. Based on this assumption, NO-sulindac may not enhance normal tissue effects of radiotherapy, protecting these tissues from radiation damage. However, experimental proof of the combined effect of NO-sulindac and radiation on the rectal wall, bladder neck and prostatic urethra is required.

Hypoxia induces a more aggressive cancer cell phenotype, resistant to radiotherapy, by upregulation of the hypoxia response [1]. HIF-1 is the transcription factor acting as the master regulator of the hypoxia response; the HIF-1 α component is most important in this regulatory process [1]. Previously we have shown that NO-NSAIDs were cytotoxic to prostate cancer cell lines under both normoxia and hypoxia and causes a post-transcriptional reduction in HIF-1 α nuclear protein [7]. The data presented in the current study demonstrates the effect that irradiation combined with NO-sulindac exposure inhibited the hypoxia response (using HIF-1 α production as a

1 surrogate). PC-3 cells treated concomitantly with NO-sulindac and irradiation
2 underwent a reduction in HIF-1 α expression over-and-above that seen with irradiation
3 or NO-sulindac alone. As such, under the conditions used in these experiments,
4 radiation treatment $\pm$ NO-sulindac may be acting by inhibition of the hypoxia
5 response, a mechanism which has been shown previously to have a synergistic cell
6 killing effect with irradiation [32, 33].

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DNA is the most important cellular target for the lethal effects of ionising radiation, with DSBs proposed to be the principal lesions responsible for radiogenic cell killing [34]. Results from both the Comet assay and γ -H2AX experiments suggest that an increase in extent of strand break damage is a possible radiobiological mechanism by which NO-sulindac may mediate radiosensitisation. Further studies using the Comet assay showed a reduction in the extent of strand break repair when the PC-3 cells were irradiated in the presence of NO-sulindac. Additionally, there was no significant decrease in the extent of γ -H2AX between 1 and 2 hours post irradiation whilst there was a significant decrease noted in the irradiated control cells; this concurs with longer repair times for DSBs (measured as γ -H2AX foci) noted for V79 cells irradiated in the presence of nitric oxide [22]. Previous studies have shown that higher levels of strand break formation and/or poorer repair, assessed by both the alkaline Comet assay and γ -H2AX foci, correlate with radiosensitivity in a variety of cancer cells, both *in vitro* and *in vivo* [35-37].

Being a relatively stable free radical species and reactive towards other free radicals, nitric oxide can be considered an 'oxygen mimetic'. Indeed, as a hypoxic sensitiser, NO seems to be significantly more efficient than oxygen [22]. However, compared to oxygen, NO has a relatively low electron affinity and the unpaired electron is able to pair up with other DNA radical species (i.e. DNA base radical

species) forming stable adducts (in combining with DNA base radical species this could render the latter incapable of undertaking hydrogen abstraction from a sugar which can lead to strand breaks [38]). In spite of these key differences, NO enhances the yields of radiation-induced SSBs and DSBs (this study & [22]), as do oxygen and nitroimidazoles [39]. The mechanism of the NO-mediated enhancement of strand break is not clear, but might involve the attempted repair processing/excision of the adducted base; indeed, this may account for the noted slower repair of both SSB (this study) and DSB [22] for cells irradiated in the presence of NO or NO-sulindac compared with cells irradiated in air (this study) or anoxia [22]. Overall, the radiobiological assay results presented above further support the discovery that NO-sulindac radiosensitises PC-3 cells.

In summary, these 2D and 3D studies have shown that NO-sulindac radiosensitises prostate cancer epithelial cells but not prostate stromal cells *in vitro*, under normoxia and to a greater extent chronic hypoxia. Plausible mechanisms for this effect being the enhanced formation and reduced repair of radiation-induced DNA strand breaks and inhibition of the radioresistant hypoxia response. These data and that from other studies [40] lend support to future clinical trials of NO-sulindac plus radiotherapy in men with prostate cancer. Ultimately, NO-NSAIDs and other NO-donors may be useful as neoadjuvant and concurrent treatments for men undergoing radiotherapy for prostate cancer.

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Prostate cell type	Oxygen conditions	Additive	D ₁₀ (Gy)
PC-3	Normoxia	DMSO	3.9
		Sulindac	4.2
		NO-sulindac	3.2
	Hypoxia	DMSO	4.4
		Sulindac	3.9
		NO-sulindac	3.1
Stroma	Normoxia	DMSO	4.3
		Sulindac	4.6
		NO-sulindac	4.5
	Hypoxia	DMSO	5.1
		Sulindac	4.9
		NO-sulindac	5.0

Table 1. Radiation dose required to reduce clonogenic survival to 10% (D₁₀ values) for PC-3 epithelial cells and primary stromal cells following incubation under normoxia or chronic hypoxia with different drug additives.

Figure legends

Figure 1. Clonogenic assay derived survival curves for PC-3 cells following incubation under normoxic or hypoxic conditions and treatment with NO-sulindac, sulindac or DMSO control showing the radiosensitising effect of NO-sulindac. (A) PC-3 cells were incubated under normoxic or hypoxic conditions prior to irradiation, at 8Gy hypoxia induced radioresistance. PC-3 cells were incubated with NO-sulindac, sulindac or DMSO under normoxia (B) or hypoxia (C) prior to irradiation. NO-sulindac radiosensitised PC-3 cells under both normoxia and hypoxia at all radiation doses. Bars, SD; *, $P < 0.05$ compared with normoxia (A) (t-test) or DMSO treated controls (B&C) (ANOVA).

Figure 2. Clonogenic assay derived survival curves for prostate stromal cells following incubation under normoxic or hypoxic conditions and treatment with NO-sulindac, sulindac or DMSO control demonstrating resistance to radiosensitisation. (A) Incubation of stromal cells under normoxia prior to irradiation did not cause significant radiosensitisation compared with hypoxic incubation. Stromal cells were incubated under normoxia (B) or hypoxia (C) with NO-sulindac, sulindac or DMSO prior to irradiation. NO-sulindac only caused radiosensitisation at 8Gy under normoxia. Bars, SD; *, $P < 0.05$ compared with DMSO treated controls (ANOVA).

Figure 3. NO-sulindac and irradiation treatment caused a reduction in the diameter of 3D sphere of PC-3 cells, this effect was maximal under hypoxia. PC-3 cells were treated with 0.05% DMSO vehicle control, 25 μ M sulindac or 25 μ M NO-sulindac and incubated under (A&C) normoxia or (B&D) hypoxia for 48 hours prior to irradiation. 3D cultures were then established. The graph bars represent the median value. Scale bar, 60 μ m. Magnification x400.

Figure 4. HIF-1 α protein levels were reduced by NO-sulindac and irradiation under normoxia and hypoxia. PC-3 cells were treated with (A) DMSO vehicle control or (B) NO-sulindac and incubated under normoxia or hypoxia for 48 hours prior to irradiation at 2Gy, the cells were incubated (at same oxygen level as pre-irradiation) for a further 8 to 48 hours prior to lysis. PC-3 cells were treated under (C) normoxia or (D) hypoxia in the presence of DMSO, NO-sulindac or sulindac for 48 hours prior to irradiation. Following irradiation the PC-3 cells were incubated (at same oxygen level as pre-irradiation) for a further 4 hours prior to lysis. There was a serial reduction in HIF-1 α with time from radiation and radiation dose. HIF-1 α production was completely abrogated by the addition of NO-sulindac. In absence of radiation there were no differences in HIF-1 α levels from 0 to 48 hours (data not shown). DMSO, 0.05% DMSO; NO-sul, 25 μ M NO-sulindac; sul, 25 μ M sulindac; Gy; RTx, radiation treatment.

Figure 5. NO-sulindac stimulates the formation of radiation-induced SSBs and DSBs and reduces DNA repair in PC-3 cells. (A) Irradiation of PC-3 cells to 4Gy in the presence of NO-sulindac significantly increases the extent of radiation-induced SSB formation compared to the control treated PC-3 cells, assessed by Comet assay. The extent of SSB formation noted upon irradiation (4Gy) of PC-3 cells in the presence of sulindac is not significantly different from the extent of SSB formation noted in the control PC-3 cells. There is no significant difference in the level of SSBs for PC-3 cells in the presence of NO-sulindac or sulindac compared to control cells at 0Gy or 2Gy irradiation. (B) Irradiation of PC-3 cells to 4Gy in the presence of NO-sulindac significantly increases the level of radiation-induced γ -H2AX foci measured 1 and 2 hours post-irradiation compared to control treated PC-3 cells. The extent of γ -H2AX foci formation noted 1 hour after irradiation of PC-3 cells in the presence of sulindac

is not significantly different from the extent of foci formation noted in the control PC-3 cells. However, the extent of γ -H2AX foci formation noted 2 hours after irradiation of PC-3 cells in the presence of sulindac was significantly different from the extent of foci formation noted in the control PC-3 cells, this being due to a significant decrease in the extent of γ -H2AX foci between 1 and 2 hours noted in the control PC-3 cells irradiated in the presence of DMSO ($P < 0.001$, ANOVA). There was no significant difference the extent of γ -H2AX foci noted at 1 and 2 hours after irradiation for PC-3 cells irradiated in the presence of either NO-sulindac or sulindac. (C) Irradiation at 8Gy and repair incubation in the presence of NO-sulindac significantly reduces the rate of radiation-induced SSB repair compared to DMSO and sulindac treated cells, except at 60 minutes. The rate of SSB repair following irradiation and repair incubation of PC-3 cells in the presence of sulindac is not significantly different from the rate of repair noted in the control PC-3 cells. *ns*, not significant; *, $P < 0.001$ (ANOVA); †, $P < 0.05$ (ANOVA).

Figure 1

Figure 2

Figure 3

Figure 4

NO-sulindac radiosensitises hypoxic PC-3 cells via DNA strand breaks and hypoxia response inhibition