

Androgen manipulation alters oxidative DNA adduct levels in androgen-sensitive prostate cancer cells grown *in vitro* and *in vivo*

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Abbreviations used: AR, androgen receptor; DHT, dihydrotestosterone; MDA, malondialdehyde; ROS, reactive oxygen species; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; ig, intragastric; M₁dG, cyclic pyrimidopurinone N-1, N² malondialdehyde-2'-deoxyguanosine; 8-oxo-dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PSA, prostate specific antigen; ROS, reactive oxygen species.

ABSTRACT

Intracellular reactive oxygen species (ROS) may cause oxidative DNA damage, resulting in the formation of adducts such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) and the cyclic pyrimidopurinone N-1, N² malondialdehyde-2'-deoxyguanosine (M₁dG). These adducts have been associated with carcinogenesis, genomic instability and clonal evolution. We tested two hypotheses in human prostate cancer cells grown in vitro and in a xenograft model: 1. treatment of androgen-sensitive cells with DHT increases levels of oxidative DNA adduct levels; 2. flutamide, a competitive androgen receptor antagonist, prevents DHT-induced changes. Levels of M₁dG and 8-oxo-dG adducts were determined by immunoslot blot and liquid chromatography-tandem mass spectrometry. M₁dG and 8-oxo-dG levels were significantly higher than control levels in LNCaP cells exposed to supra-physiological concentrations (25 to 100 nM) of DHT (both $P < 0.05$ by ANOVA). Flutamide pre-treatment completely prevented this increase. In the xenograft model, tumor levels of M₁dG were decreased by 46% ($P = 0.001$ by Mann Whitney Test) in flutamide-treated animals compared to controls. The changes demonstrated suggest that oxidative DNA adducts may serve as biomarkers of the efficacy of androgen manipulation in chemoprevention trials.

1. Introduction

Cancer of the prostate is the most commonly diagnosed solid malignancy and the second commonest cause of cancer-related deaths in men living in developed countries.¹ In the United Kingdom, the incidence is likely to double over the next 20 years due to a combination of improved longevity of the general population and more widespread use of prostate specific antigen (PSA) testing.² It has become increasingly apparent that reactive oxygen species (ROS) may be an important aetiological factor in the development and progression of prostate cancer [reviewed in 3]. Exogenous and endogenous ROS of biological significance include hydrogen peroxide, superoxide ions and hydroxyl radicals, which may attack lipids, proteins and DNA within cells.⁴ Damage induced by ROS can modulate gene transcription and alter the expression of transcriptional factors implicated in carcinogenesis, including *c-fos* and *c-jun* oncogenes.^{5,6} A “pro-oxidant state,” measured by an increase in ROS or a disruption of protective anti-oxidant systems, has been observed in prostate cancer and in the pre-malignant lesion, high grade prostatic intraepithelial neoplasia.⁷

Levels of oxidative DNA adducts, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) and the cyclic pyrimidopurinone N-1, N² malondialdehyde-2'-deoxyguanosine (M₁dG), reflect direct and indirect oxidation of DNA by ROS.^{8,9} These adducts have been associated with mutagenesis and carcinogenesis in preclinical models [reviewed in 10]. Since “control” DNA from tissue unexposed to endogenous mutagens does not exist *in vivo*, correlative studies represent the only means of relating changes in DNA adduct levels to carcinogenic events or intervention with potentially chemopreventive agents.

Androgens play a key role in the development and progression of the majority of prostate cancers.^{11,12} Indeed, prostate cancer rarely develops in men castrated before puberty.¹³ In the androgen-sensitive human prostate cancer cell line, LNCaP, stimulation with androgens has been shown to result in increased levels of ROS.¹⁴ The same androgen stimulation of androgen-insensitive DU145 cells did not influence levels of ROS.¹⁴ In a more recent clinical study, patients with prostate cancer who underwent hormonal therapy demonstrated a significant decrease in mean urinary 8-oxo-dG levels after androgen deprivation therapy compared to mean levels measured before therapy.¹⁵ Collectively, these data suggest that oxidative stress is associated with prostate carcinogenesis and that androgen suppression may be linked to a decrease in oxidative DNA damage. Such damage may contribute to the development of the malignant phenotype in pre-malignant lesions or to genomic instability in established tumours which may feed the clonal evolution of the cancer.^{16,17}

In view of these results, we postulated that androgen stimulation increases levels of oxidative DNA adduct levels in androgen-sensitive prostate cancer cells and that flutamide, a competitive androgen receptor antagonist, prevents androgen-induced oxidative DNA damage. We tested these hypotheses in two preclinical model systems: LNCaP cells grown *in vitro* and the same cell line grown as a xenograft in athymic male mice.

2. Materials and methods

2.1 Cells and incubations

Human-derived malignant prostate cell lines, LNCaP, DU145 and PC3 were obtained from the European Collection of Cell Cultures (Salisbury, UK) and cultured in large

flasks in RPMI 1640 (25 mM HEPES) with 1% sodium pyruvate (Sigma-Aldrich, Poole, UK) and 1% glutamax (Gibco, Paisley, UK) containing 10% foetal calf serum. Cells of less than 20 passages were seeded either in flasks (10^6) for oxidative DNA adduct measurements, or in 12-well plates (10^4 per well) for measurements of proliferation. Since foetal calf serum contains physiological levels of androgens,¹⁸ experiments with flutamide required cells to be serum-deprived for 24 h prior to daily treatment with dihydrotestosterone (DHT) and/or the competitive androgen receptor antagonist, flutamide. DHT, flutamide and ethanol (the vehicle for the former 2 agents) were obtained from Sigma-Aldrich (Poole, UK). Cells were treated with physiological (2.5 nM) or supra-physiological (5 to 100 nM) concentrations of DHT daily. Flutamide was added 30 min prior to DHT, to give a final concentration in medium of 5 or 10 μ M. The DHT and flutamide concentrations employed in these experiments were based on those shown to have pro-oxidant and antioxidant activity in LNCaP and DU145 cells in previous studies.^{14,19,20} Control cells were exposed to the equivalent volume of ethanol. Cells were harvested using trypsin (1%).

2.2 *Animals and treatments*

Experiments were carried out under animal project licence PPL 40/2496 granted to the University of Leicester by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards set by the revised guidelines from the UK Coordinating Committee on Cancer Research (UKCCCR, London, UK). Male MF-1 outbred nude mice (30–40 g), obtained from Harlan UK (Bicester, Oxon, UK), were ear-punched for identification and maintained at 20–23°C under conditions of 40 – 60% relative humidity and a 12-hour light/dark cycle. Mice received an irradiated RM3 high protein rodent diet *ad libitum* and sterilized drinking water. LNCaP tumour

cells were implanted and grown in nude mice as described previously.²¹ After 7 days of acclimatization, animals were subjected to light anaesthesia with halothane (Concord Pharmaceuticals, Dunmow, UK), and LNCaP prostate tumour cells (5×10^6) suspended in 200 μ l of matrigel (Becton Dickinson, Worthing, UK) were injected subcutaneously into the right flank. Five mice were not injected as non-tumour controls. Mice were weighed weekly and assessed for tumour growth by measurement using callipers. When tumour size reached approximately 6-7 mm in diameter, mice were tail-bled to obtain blood ($\sim 100 \mu$ l) for serum PSA measurement. At this time-point, mice (12 per group) were randomized to either treatment or control group. Mice received flutamide (50 mg/kg, formulated in 1.25% ethanol/water, dosage volume 3.33 mL/kg) *via* the *ig* route daily for 7 days. This dose was based on previous dose finding studies of flutamide in rodents.^{22,23} Mice in the control group received the same volume of the vehicle *ig*. Mice that did not develop tumours were separated into control and treated groups ($n = 5$ each group) and dosed as described above. Twenty four hours after the seventh daily dose, tumour size was re-measured, mice were re-weighed and then ex-sanguinated under terminal halothane anaesthesia (cardiac puncture). Healthy prostate and tumour tissue were excised, weighed and frozen (-80°C) prior to manual homogenization and DNA extraction (*vide infra*). Serum was obtained from blood by centrifugation, frozen and stored at -80°C until analysis.

2.3 Histopathology

Some murine prostate tumours were fixed in formalin for a minimum of 2 weeks and then embedded in paraffin wax. Sections (5 μ m thick) were cut and stained with haematoxylin and eosin before microscopic examination.

2.4 Measurement of PSA, M₁dG and 8-oxo-dG DNA adduct levels

PSA in cellular supernatant or murine serum was measured by radioimmunoassay kit (3rd generation immulite assay, Diagnostic Products Corporation, CA, USA).

For oxidative DNA adduct measurement, genomic DNA was isolated from cells and murine tumour tissue by the Qiagen method (Qiagen, Crawley, UK) for analysis of M₁dG, or by the Wako DNA extractor WB kit (Wako Chemicals, Neuss, Germany) and sodium iodide (NaI) for the analysis of 8-oxo-dG. The extraction was performed according to the manufacturer's instructions. In the case of the "Wako" isolation method, additional incubations with RNase A (20 U), RNase T₁ (2 U) and proteinase K (100 µg) were included prior to the addition of NaI (all obtained from Sigma-Aldrich). Levels of M₁dG adducts in cells or murine tissues were determined in triplicate by an immunoslot blot method, as described previously,²⁴ using a murine monoclonal anti-M₁dG antibody (provided by Dr L.J. Marnett, Vanderbilt University, TN, USA) and goat anti-mouse horseradish peroxidase conjugated secondary antibody (Dako, Ely, UK). The M₁dG adduct level in each sample was corrected for the amount of DNA bound to the filter as determined by propidium iodide staining.²⁵ The detection limit for M₁dG was approximately 0.5 adducts per 10⁷ nucleotides. Experiments using cells were normalised to number of viable cells used for DNA extraction.

Levels of 8-oxodG adducts in cells were determined by an immunoaffinity column purification liquid chromatography-tandem mass spectrometry (LC-MS/MS) method as described previously.²⁶ Prior to enzymatic digestion, a stable isotope [¹⁵N₅]8-oxodG internal standard was added to each DNA sample. Following enzymatic digestion, an aliquot (1 µg) of the digested DNA was analysed by HPLC-UV to determine the level of 2'-deoxyguanosine. Each digested DNA sample was subjected to immunoaffinity column purification prior to analysis by LC-MS/MS. The

LC-MS/MS consisted of a Waters Alliance 2695 separations module with a 100 μL injection loop connected to a Micromass Quattro Ultima Pt. (Micromass, Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 110°C and the desolvation temperature at 350°C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas was set to zero. The capillary voltage was set at 3.20 kV. The cone and RF1 lens voltages were 42 V and 30 V, respectively. A HyPurity (Thermo Electron Corporation, Runcorn, UK), C_{18} , (1.0 \times 150 mm, 3 μm) column connected to a Uniguard HyPurity C_{18} (1.0 \times 10 mm, 3 μm) guard cartridge and a KrudKatcher disposable pre-column (0.5 μm) filter was eluted isocratically with 0.1% acetic acid/methanol (90:10, v/v) at a flow rate of 50 $\mu\text{L}/\text{min}$. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV. The dwell time was set to 200 ms and the resolution was one m/z unit at peak base. The samples were analysed in positive electrospray ionization MS/MS selected reaction monitoring mode (SRM) for the $[\text{M}+\text{H}]^+$ ion to base $[\text{B}+\text{H}_2]^+$ transitions of 8-oxodG (m/z 284 to 168) and $^{15}\text{N}_5$ 8-oxodG (m/z 289 to 173). The limit of detection of 8-oxodG was 2.0 fmol on the column. The level of 8-oxodG was determined in each sample from the ratio of the peak area of 8-oxodG to that of the internal standard $^{15}\text{N}_5$ 8-oxodG and normalised to the amount of 2'-deoxyguanosine observed for each sample following DNA digestion as determined by HPLC-UV.

2.5 Statistical analysis

Results were subjected to analyses of variance (ANOVA) and covariance with a 5% significance level using Minitab software (Minitab Inc., PA, USA). Plots of residuals

were used to ensure that variances were homogeneous and that the residuals had a normal distribution. Non-parametric data were analysed by Mann Whitney test.

3. Results

3.1 Oxidative DNA adduct levels in cells in vitro and effects of exposure to flutamide

Background levels of the oxidative DNA adducts, M₁dG and 8-oxo-dG were determined in LNCaP, DU145 and PC3 cells after incubation in medium containing 10% foetal calf serum (i.e. physiological levels of androgens – see above). Treatment of androgen-sensitive LNCaP cells with supra-physiological doses of DHT caused a significant increase in levels of both oxidative adducts (Fig. 1). In contrast, such an increase was not observed in the androgen-insensitive DU145 and PC3 cells. As DHT-treatment affected cell proliferation, oxidative DNA adduct levels were normalized with respect to the number of viable cells used for DNA extraction: This correction did not alter the observed effect (data not shown). Under conditions of growth in the presence of serum, both types of oxidative DNA adduct were elevated in LNCaP cells over control levels at concentrations of DHT of 25 nM and above (Fig. 2).

To test the hypothesis that androgen receptor (AR) stimulation increases oxidative DNA adduct levels in androgen-sensitive cells, LNCaP cells were deprived of serum for 24 h and then incubated daily with a physiological concentration of DHT with or without pre-incubation (30 mins) with the competitive AR antagonist, flutamide. Flutamide counteracted the increase in levels of M₁dG or 8-oxo-dG caused by DHT (Fig. 3). In fact, 10 μ M flutamide completely abrogated the oxidative DNA adduct level increase caused by 100 nM DHT. In these experiments, it should

be noted that flutamide affected LNCaP cell growth and generation of PSA after 24 h: Flutamide (10 μ M) reduced the growth of LNCaP cells grown in the presence of DHT (100 nM) by 70% and it reduced PSA in the supernatant of cells cultured in the presence of serum by 85%. Correction of adduct levels with respect to the number of viable cells used for DNA extraction did not alter the effect demonstrated (data not shown).

3.2 Effect of flutamide on LNCaP tumor growth, serum PSA and oxidative DNA adduct levels in vivo.

To extend the results obtained in cells *in vitro* to a rodent model in which the same cancer cells could be grown under physiological conditions *in vivo*, experiments were conducted in athymic nude mice bearing a LNCaP xenograft. Seventy percent of the mice that received LNCaP cells subcutaneously developed measurable tumours. Compared to control mice, the presence of tumour did not affect murine bodyweight. Treatment with flutamide (50 mg/kg *ig* per day) for 7 days reduced mean tumour size and mean serum PSA concentration significantly, compared to animals which received vehicle only (Fig. 4). In the mice that did not develop measurable tumours, PSA was undetectable. When tumour tissue was subjected to pathological investigation, tissue from animals on flutamide displayed areas of focal necrosis, which were absent from mice which had received vehicle only (Fig. 5), consistent with an anti-neoplastic effect of flutamide.

M₁dG adduct levels were measured in tumour tissue, normal prostate tissue and peripheral blood cells. As shown in figure 6, administration of flutamide almost halved M₁dG levels in the tumour, whereas M₁dG levels in peripheral blood cells and benign prostate tissue were unaffected by flutamide treatment.

4. Discussion

The results shown here provide the first reported description of oxidative DNA adduct levels in human malignant prostate cells grown *in vitro* and as a solid tumour in mice *in vivo*. M₁dG formation can result from the reaction of malondialdehyde (MDA) with DNA, but other sources have been described.⁸⁻¹⁰ In the absence of lipid peroxidation, oxidation of DNA by bleomycin *in vitro* gave rise to M₁dG adducts via base propenal formation.⁸ Site-specific mutagenesis studies have suggested that mutation frequency caused by indirectly formed oxidative DNA adducts, such as the M₁dG adduct, is similar to that of directly formed oxidative DNA adducts, such as 8-oxo-dG.²⁷ Moreover, clinical data have also indicated a relative insensitivity of M₁dG adduct levels to changes in MDA concentration in human tissues, when compared to the association of M₁dG adduct levels with 8-oxo-dG levels.^{9,28} Based on these collective preclinical and clinical data, M₁dG adduct levels are currently considered biomarkers of oxidative DNA damage that reflect levels of ROS.²⁹ The correlative data for M₁dG and 8-oxodG adduct levels presented in the study described here provide further support for this hypothesis.

The LNCaP cells used in the study presented here were susceptible to the antagonist activity of flutamide, as reflected by cell growth inhibition and reduction in PSA production. Flutamide prevented increases in oxidative DNA adduct levels induced by supra-physiological concentrations of DHT. Although DHT levels vary within individuals (with circadian variation), between individuals and between tissues of one individual, a recent study of serum androgen levels in 132 men aged 30-72 years demonstrated a maximum titre of serum DHT around 2.0 nM with an inter-

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individual standard deviation of 0.15 nM.³⁰ These data are compatible with the findings of previous studies in which different assay techniques have been used and slightly higher means have been obtained.^{31,32} Collectively, it can be stated that concentrations of DHT above 10 nM are regarded as above the physiological level observed in the serum of men. On account of the limitations of the cell culture model in reproducing low nanomolar concentrations of DHT in physiological circumstances, LNCaP cells were grown as xenografts on nude mice. In this model system, flutamide significantly reduced M₁dG adduct levels when administered at a dose which impeded tumour development and PSA generation, and the treatment caused tumour tissue necrosis (figure 5). The level of M₁dG adducts in the LNCaP tumours of untreated animals was similar to levels of adducts in cells grown *in vitro* stimulated with DHT.

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The findings of the study presented here are compatible with the demonstration that stimulation with androgens results in increased levels of ROS in androgen-sensitive LNCaP cells.¹⁴ Although it is not currently clear if androgen stimulation also affects detoxification enzymes for ROS in these cells, preliminary data from a xenograft model using androgen-responsive PC3 cells suggest that androgens increase levels of ROS-detoxifying enzymes and conversely androgen deprivation sensitises cells to the toxic effects of ROS.³³ These findings are compatible with the findings of an earlier study in male rats in which castration was associated with upregulation of certain ROS-detoxifying enzymes.³⁴ Although definite conclusions cannot be drawn, it currently appears that the link between androgen stimulation and increased oxidative DNA adduct levels in androgen-sensitive cells is more likely to be attributable to increased generation of ROS rather than diminished detoxification. There are no data currently available on potential

effects on specific DNA repair enzymes, such as 8-oxoguanine DNA glycosylase (OGG1), involved in the repair of oxidative lesions.

It is noteworthy that, although a significant change in M₁dG levels was demonstrated in the LNCaP tumours *in vivo*, M₁dG levels in normal prostate tissue under the same conditions were not affected. This discrepancy may relate to differences between normal and malignant prostate tissue in cellular responses to oxidative DNA damage. A recent study of human prostate tissue from patients with benign prostatic hypertrophy and prostate cancer offered further insight into the link between carcinogenesis and oxidative DNA damage.³⁵ In this study, levels of repair proteins and 8-oxo-dG adducts were analyzed by Western blot and immunochemistry respectively. The results suggested that oxidative DNA damage may drive neoplastic cells to activate repair mechanisms which favour escape from senescence and consequential clonal expansion of DNA-damaged clones, whereas increased oxidative DNA damage in non-malignant prostate cells may result in senescence. Analysis of human precancerous lesions indicates that DNA damage response and senescence markers co-segregate,³⁶ suggesting that senescence and apoptosis in human preneoplastic lesions provide barriers to malignant progression and that malignant cells have acquired methods to overcome such barriers.

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A potential link between androgen manipulation and levels of oxidative DNA damage has been suggested by Miyake et al,¹⁵ who used a quantitative enzyme-linked immunosorbent assay to measure oxidative DNA adduct levels in urine samples from 82 patients with prostate cancer (with correction for urinary creatinine levels), 40 of whom underwent hormonal manipulation. Although radical prostatectomy in 42 patients did not significantly affect levels of 8-oxo-dG in the pre- and post-surgical urine samples, in patients who received treatment with luteinizing hormone-releasing

hormone analogue injections and/or an anti-androgen, mean urinary 8-oxo-dG levels diminished by approximately 20% ($P < 0.05$ by Mann Whitney test). Since the detection of a metabolite of M₁dG by LC/MS has recently been reported in urine from healthy volunteers, corroborated by nuclear magnetic resonance spectroscopy and independent chemical synthesis,³⁷ the detection of oxidative DNA adducts and their oxidative metabolites is currently regarded as a promising opportunity for the clinical development of biomarkers of endogenous DNA damage associated with inflammation, oxidative stress and carcinogenesis. It is important that highly specific and sensitive analytical methods are used for the analysis of urine samples, such as isotope-dilution LC-MS/MS.³⁸

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The clinical data from the study of Miyake et al¹⁵ and the results from the xenograft model presented in the study reported here are consistent with the hypothesis that the principal anti-oxidative effect of hormonal treatment is in malignant prostate tissue. Further support for this hypothesis is provided by a phase II trial performed in men undergoing radical prostatectomy for prostate cancer who received dietary intervention in the form of supplements of tomato sauce containing high levels of the anti-oxidant, lycopene for 3 weeks prior to surgery.³⁹ The investigators demonstrated that post-intervention levels of 8-oxo-dG, measured by HPLC with electrochemical detection, were significantly reduced in prostatic tissue compared to pre-intervention levels in the same patients. This trial indicates that measurable levels of oxidative DNA adducts are present in human prostate tissue and that levels may be influenced by dietary supplementation over a short time frame.

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The results presented in the study reported here extend these findings to suggest that oxidative DNA adducts in malignant prostate tissue may reflect a mechanistic link between androgen sensitivity and DNA damage. If future studies

corroborate such an association in humans *in vivo*, oxidative DNA adduct levels could be developed as potential biomarkers of the efficacy of androgen manipulation in the chemoprevention studies of androgen manipulation which are currently following the precedent set by the Prostate Cancer Prevention Trial⁴⁰

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Figure legends

Fig 1 - Effect of daily treatment with DHT (100 nM) on levels of M₁dG (A) or 8-oxo-dG (B) in DU145, PC3 and LNCaP cells grown in medium.

Open bars denote untreated cells and closed bars denote treated cells. Cells (approximately 10⁶) were seeded in flasks and grown for 3 days in medium containing 10% foetal calf serum. DHT was then added daily for 7 days. Results (mean + SD) are presented from three separate experiments. Asterisks indicate that the difference is significant compared to control cells ($P < 0.05$ by ANOVA in A; $P < 0.01$ by Mann Whitney test in B). For details of cell culture and measurement of M₁dG and 8-oxo-dG, see Materials and methods.

Fig 2 - Concentration dependence of effect of DHT on levels of M₁dG (A) or 8-oxo-dG (B) in LNCaP cells.

Oxidative DNA adduct levels in incubations of cells omitting DHT are denoted “control” (open bars). Cells (approximately 10⁶) were seeded in flasks and grown for 3 days in medium containing 10% foetal calf serum. DHT was then added daily for 7 days. Results (mean + SD) are from 3 experiments. Stars indicate that the difference to respective control cells is significant ($P < 0.05$ by ANOVA). For details of cell culture and M₁dG or 8-oxo-dG measurements, see Materials and methods.

Fig 3 - Effect of flutamide (10 μM) on increase in M₁dG (A) or 8-oxo-dG (B) caused by DHT (100 nM) in LNCaP cells.

Oxidative DNA adduct levels in incubations of cells omitting DHT are denoted “control” (“C”, open bars). Cells (10⁶) were seeded in flasks and grown for 3 days in

medium containing 10% foetal calf serum. Unlike the data shown in figures 1 and 2, cells were then grown in serum-free medium with or without the daily addition of flutamide and/or DHT for 4 days. Results (mean + SD) are from 3 experiments. Stars indicate that difference to control cells is significant, crosses indicate that the difference to cells incubated with DHT alone is significant ($P < 0.05$ by ANOVA). C=control; Flut = flutamide. For details of cell culture and M₁dG and 8-oxo-dG measurements, see Materials and methods.

Fig 4 - Effect of treatment with flutamide (50 mg/kg *ig*) daily for 7 days on tumour volume and serum PSA in athymic MF1 mice bearing the LNCaP tumour.

Open bars: control mice. Closed bars: treated mice. Results are presented as percentage change from pre-dosing volume/levels. In untreated animals, the mean terminal tumour volumes and PSA concentrations were $506 \pm 476 \text{ mm}^3$ and $57 \pm 31 \text{ ng/mL}$ (mean \pm SD, $n = 12$), respectively. When original pre- and post-dose values were compared, the difference was statistically significant ($P < 0.001$ by ANOVA) for both types of measurements. For details of tumour implantation and analyses of tumour volume and PSA, see Materials and methods.

Fig 5 - Representative photomicrographs of LNCaP tumours from control mice (A) and mice that received flutamide (50 mg/kg *ig*) daily for 7 days (B).

Haematoxylin and eosin stain, magnification x 250. Areas of focal necrosis and cell debris are demonstrated by arrows in B.

Fig 6 - Effect of treatment with flutamide (50 mg/kg *ig*) daily for 7 days on M₁dG levels in xenografted prostate tumour, normal prostate tissue and blood leukocytes from non-tumour-bearing mice or mice bearing LNCaP tumours.

Open bars: control tumour-bearing mice (treated with vehicle only). Hatched bars: non-tumour-bearing mice (treated with vehicle only). Closed bars: tumour-bearing mice treated with flutamide. Results are mean \pm SD from 12 mice. Stars indicate that the difference to control mice is significant ($P < 0.001$ by Mann Whitney test). For details of tumour implantation and measurement of M₁dG levels, see Materials and methods.