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PRINCIPAL INVESTIGATOR: Orla A. O'Mahony, Ph.D.

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, MI 48109-1274

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14. ABSTRACT We proposed to study the mechanisms of androgen resistance by focusing on androgen receptor mutations that arise due to the selective pressures of antiandrogen treatment. In vitro we examined the mechanistic actions of bicalutamide and flutamide in transfection studies in a variety of cell lines and on different androgen responsive promoters. We have shown that flutamide exhibits agonist action in a cell specific manner. In vivo we utilized two models to test our hypothesis, xenograft models of human prostate cancer cell lines and a novel transgenic mouse model that expresses a humanized androgen receptor in the TRAMP background. We are currently assessing tumor progression in both models and sequencing AR cDNAs from tumors with different endocrine status.					
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Introduction

Prostate tumour growth and progression initially depend on androgen and consequently tumours regress in response to androgen ablation therapy. Unfortunately, prostate tumours inevitably gain the ability to circumvent androgen ablation treatments and become resistant to therapeutic intervention. This occurs despite the continued presence of the androgen receptor (AR) in late stage prostate tumours and in spite of evidence that suggests that the androgen-signalling pathway remains intact (Buchanan *et al.*,2001 Zegarra-Moro *et al.*,2002). One potential mechanism for anti-androgen resistance is that mutations in AR provide the tumour with a growth/survival advantage in this androgen depleted environment.

We propose that selection of mutant ARs may be treatment specific and that genetic alterations in AR occur as a direct consequence of the specific selection pressures applied to the prostate tumour during androgen ablation therapy. Preliminary evidence from our laboratory indicates that there are subtle differences in the action of bicalutamide and flutamide on AR function, particularly in relation to partial agonist activity and coactivator interaction. Comparison of spontaneous AR mutations in tumours from castrated and intact transgenic adenocarcinoma mouse prostate (TRAMP) mice indicates that AR mutations cluster in distinct regions dependant on the hormonal status of the animal (Han *et al.*, 2000). To date no study has compared the possible differences in selection pressure applied by bicalutamide and flutamide on the AR function *in vivo*. This information has clinical significance, as AR mutations resistant to one antagonist may not be resistant to the second.

To investigate agent specific selection pressure on AR function we proposed to identify AR variation in tumours excised from prostate cancer cell line xenograft mouse models treated with either bicalutamide or flutamide. We will characterize novel AR variants *in vitro* to assess the functional significance of the AR mutation.

Characterization of molecular changes in AR associated with androgen independent progression would facilitate the optimization of prostate cancer therapy and prevention based on AR sequence and structure. Greater understanding of the functional behaviour of AR mutants that arise during prostate cancer treatment may provide critical information for the development of stage-specific treatments, as well as highlighting novel targets for therapeutic intervention.

The aims outlined in the Proposal were as follows:

Specific Aims

1. *To compare the mechanistic actions of bicalutamide and flutamide in prostate cancer cells in vitro.*
2. *To use PC-3 and VCaP xenograft mouse models to test the hypothesis that different treatment regimes (no treatment, bicalutamide, flutamide, in the presence and/or absence of hormone) selects for treatment-specific AR variants.*
3. *To characterize functionally AR molecular variants that may result from antiandrogen treatment of the mouse xenograft models and to test their capacity for tumour promotion.*

We have focused our efforts on aim 1 and 2. Our research strategy outlined below, has been modified in accordance with reviewer's comments as well as preliminary data obtained.

BODY

Task 1: -To compare the mechanistic actions of bicalutamide and flutamide in prostate cancer cells *in vitro*.

Rationale

Advanced prostate cancer patients often respond to a second line of antiandrogen therapy (Miyake *et.al.* 2005). It has also been illustrated that antiandrogens can have differing effects on the interactions between AR and co-activator molecules (Song *et al.*,2004). This suggests that different antiandrogens may have alternate downstream signalling mechanisms, which may ultimately result in differential modes of prostate disease progression. Our hypothesis suggests that bicalutamide and flutamide will have subtly different effects on AR function that may be accentuated by different promoter and cell-context effects.

Experimental Design

We proposed to carry out a series of transfection studies involving an array of different cell lines (CV-1, PC3, ^{PC3-HAR}, ^{PC3-LNCaP AR}) and two androgen responsive target elements (3xHRE, PSA) to reveal promoter and cell-context dependent differences in antagonist action.

Results/Discussion

To explore potential cell context effects on antiandrogen action we initially focused on two androgen receptor negative cell lines, CV-1 and PC 3. CV-1, a monkey kidney fibroblast cell line is utilised extensively in transfection studies while the PC3 cell line is representative of an advanced prostatic carcinoma isolated from a lumbar vertebral metastasis. The PC3 cell line has been used widely in the field of prostate cancer and exhibits androgen independent tumour growth both *in vitro* and *in vivo*.

Both cell lines were transfected with 4 ng pSG5 human androgen receptor expression vector and 20 ng CMV-renilla vector which was used as a transfection control. Two contrasting target reporter genes were utilised; a simple promoter containing three tandem copies of a consensus hormone response element (3XHRE) and a complex prostate specific promoter, PSA luciferase, which contains an upstream androgen response enhancer region and the PSA proximal promoter.

Examination of flutamide action in CV-1 cells on both simple and complex promoters illustrated an agonist effect at a higher concentration (Fig 1 C and D). Flutamide agonist action was not observed on either the simple or complex promoter in PC3 cells. Flutamide agonist action has been previously observed, and in fact antiandrogen withdrawal syndrome has been attributed in part to the potential agonist action of flutamide. *In vitro*, flutamide agonist activity was demonstrated in LNCaP cells which express a mutated AR (T877A) which allows for promiscuous activation of the receptor by other steroid hormones, as well as agonist activation by flutamide.

To fully explore differences in antiandrogen action we also proposed the use of the cell lines, PC-3^{hAR} and PC-3^{LNCaP AR}. The PC-3^{LNCaP AR} was generated in our laboratory by stable

transfection of a mutated AR (T877A) to mimic the LNCaP mutated receptor. The PC-3^{AR} cell line was a kind gift from Dr. Burnstein, University of Miami School of Medicine, FL, US and contains a stably transfected wild type AR. Published data, as well as evidence produced in our laboratory, indicated the androgen responsiveness of these cell lines. In the case of the PC-3-hAR cell line, specific androgen binding and immunoblotting confirmed the expression of AR protein, and androgen transactivation of the MMTV luciferase reporter was also observed (Heisler *et al.*, 1997). Transfection studies of PC-3- LNCaP AR have shown the ability of the LNCaP AR to induce a luciferase reporter system (see Fig. 2). Androgen stimulation of both PC-3-hAR and PC-3^{LNCaP AR} cell lines illustrated altered AR expression on DHT stimulation, in agreement with previous studies (Data not shown, Dai *et al.*, 1996). Transfection studies with these cell lines are ongoing.

TASK 2: - To examine whether PC-3 and VCaP xenograft mouse models treated with different therapy regimes can result in agent-specific AR variants.

Rationale:

The primary hypothesis of this proposal is that different treatment regimes can select for specific variant ARs that offer a growth advantage to PCa tumours. TRAMP mice have indicated that mutations found in castrated mice segregate to the N-terminal region of AR but those identified in intact mice were located to the LBD suggesting hormonal influence on the type of AR mutations (Han *et al.*, 2001). Mutation analysis of bicalutamide treated patients has also highlighted mutations that were not observed in flutamide-treated patients (Haapala *et al.*, 2001). Recently tissue removed from a patient treated with bicalutamide only exhibited tumour growth in SCID mice in the presence of bicalutamide. Sequence analysis identified the mutation at W741C (Yoshida T *et al.*, 2005).

Experimental Strategy:

It was proposed to generate mouse xenograft models by implantation of four cell lines PC-3^{hAR}, PC-3^{LNCaP-AR}, VCaP and PC-3 into SCID mice. It was expected that hormonal manipulation of these mice, by castration and antiandrogen treatment, would provide different selection pressures for variant ARs. The VCaP cell line was isolated from a prostate cancer vertebral metastasis of a flutamide treated patient. VCaPs express AR and have shown androgen sensitive growth both *in vivo* and *in vitro* (Korenchuk *et al.*, 2001). The PC-3 cell lines utilized to generate xenograft models are discussed above.

Results/Discussion

We anticipated that utilization of xenograft models would allow us to establish whether **1)** prior treatment with a particular antiandrogen (as in VCaP cell lines) would influence the selection of AR variants produced when compared to the PC-3 sublines **2)** the presence of mutation in the AR (PC-3^{LNCaP}) alters tumourigenicity when compared to PC-3^{AR} which contains wild type AR, **3)** agent-specific AR are generated subsequent to different treatment regimes.

We initially proposed to analyze bicalutamide and flutamide treated mice. Since previous studies in TRAMP mice have indicated that castration can effectively generate hormone specific AR

variants we considered it prudent to test our model initially with castration prior to hormonal manipulation with specific antiandrogens.

Examination of the VCaP cell line xenografts illustrated that the growth of tumour cells in castrated SCID mice was slower than that observed in intact mice (fig 3). Immunocytochemistry illustrated that all castrate tumours were AR positive and qualitative analysis of immunoblotting indicated no difference in levels of AR expression (Fig. 3). RNA was extracted from tumours using Qiagen RNeasy columns, reverse transcribed using oligo dT primers and aliquots were subjected to PCR with a proofreading polymerase. For complete analysis of the coding region of androgen receptor, 5 specific primer pairs were designed that divided the AR into five overlapping segments (Fig. 4). PCR products were cloned into pGEM TA vector (Promega), transformed into DH5 α competent cells and 10 colonies were picked and prepared for sequencing for each tumour and for each PCR product. All DNA samples were sequenced at the University of Michigan Sequencing core and analysed using Sequencher software (Gene Codes Corporation). To control for variation due to reverse transcriptase and/or taq polymerase errors, two RT reactions will be performed per tumour and 10 cDNAs subcloned and sequenced for each reaction. We are in the process of analysing the second set of clones to confirm mutations identified in Fig. 4.

Examination of tumour growth of the PC3 cell line implants showed no measurable difference in tumour growth between castrated and intact mice. Although the growth rate differed between cell lines, this may reflect their *in vitro* growth rates rather than the hormonal manipulation. Lack of response to androgen withdrawal was expected of the PC3 parental cell line, as this line has been repeatedly shown to be AR negative (Van Bokhoven *et al.*, 2005, Heisler *et al.*, 1997). It was surprising that xenografts generated using the PC3 cell lines stably transfected with either wild type AR or the mutated LNCaP AR exhibited no alteration in tumour growth rate in the presence or absence of androgen (Fig. 5). Despite the observed androgen responsiveness of these cell lines *in vitro* (Fig. 2), *in vivo* PC3-hAR and PC3^{LNCaP AR} cell failed to exhibit any alteration of tumour growth on androgen withdrawal. It may be suggested that this lack of androgen responsiveness *in vivo* is a characteristic of the parental cell line. The PC3 parental cell line lacks expression of functional AR and has already achieved the capability to proliferate in the absence of androgen. Consequently, *in vivo* stimulation of an alternative androgen independent growth signaling pathway in the xenografted tumour cells may override any potential influence the stably transfected AR may have on proliferation. It may also be suggested that the expression of AR in PC3 stably transfected cell lines may be too low to exhibit potent androgen responsiveness *in vivo*.

Therapeutic failure and development of androgen independent disease despite the presence of the AR is a major problem in treatment of prostate cancer and it appears that PC3^{-hAR} and PC3^{LNCaP} xenografts may be an illustration of this late stage of prostate cancer.

The lack of effect of castration on tumour growth in PC3, PC3^{-hAR}, PC3^{LNCaP} strongly indicates that these xenografts might not be appropriate to investigate the potential selection pressures of the antiandrogens *in vivo*.

Alternative Experimental Strategy

Rationale

Another major focus of research in our laboratory is how genetic variation in AR affects the initiation and progression of prostate cancer. To study the influence of AR polymorphisms on prostate cancer initiation and progression, mouse strains carrying human AR N-terminal sequences in place of the mouse N-terminal were generated. Since the N-terminal region of the human AR is most divergent from mouse AR, this strategy generated a strain of mice with a 'humanized' AR (hAR). Crossing the hAR mouse to a prostate cancer mouse model, TRAMP, resulted in mice that show similar characteristics of human PCa progression, with PIN lesions arising at 12 wks of age. This model offers the unique opportunity to investigate the human AR in an in vivo setting, but in a homogenous genetic background. Consequently we proposed to test our hypothesis that prostate treatment can provide a selection pressure on production of AR mutation using these hAR xTRAMP mice. The proposed experimental strategy is highlighted in Fig. 6

The hAR x TRAMP mice (10/group) will begin treatment with antiandrogen (Flutamide 25 mg/Kg and Casodex 50 mg/Kg) at 12 wks, or on tumour initiation as detected by abdominal palpation. Published data indicates that at 12 wks TRAMP mice exhibit PIN, considered to be a precancerous lesion of the prostate. MRI as well as weekly assessment for palpable tumours will assess tumour initiation. Analysis of tumour growth and treatment length differences between flutamide and casodex will be observed. Once mice become moribund, necropsy will be carried out and tumours harvested and prepared for AR sequence analysis as outlined previously.

To date mice have been assigned to all groups. Tumours have been harvested from 90% of the flutamide and of the casodex treated groups. Mouse breeding is ongoing to fill groups that will be treated at the onset of PIN, at 12 weeks.

Both antiandrogen treatments have been shown to be effective as endocrine treatment slows tumour progression (Fig. 6). No significant difference in treatment length is observed between tumours treated at the time of detection of a palpable tumour with flutamide or bicalutamide.

Interestingly preliminary examination of tumour growth in mice treated with antiandrogens at an early time point, before onset of palpable tumour, highlights a dramatic difference in length of disease course between antiandrogen therapy and castration. This may suggest a difference in mechanisms of AR action on antiandrogen treatment. When the AR ligand-binding pocket is occupied with antiandrogens, different mechanisms of AR downstream signaling may occur when compared to castration when, in mice, the ligand binding pocket is potentially empty. Further analysis is required to elucidate the mechanisms underlying this difference in tumour behavior.

Key Research Accomplishments

- We generated xenograft model systems in which the VCaP xenografts showed androgen dependant growth but PC3 cell lines did not.
- We are in the process of verifying unique AR mutations in VCaP cell line xenografts.
- The treatment of the hAR x TRAMP mouse with antiandrogens slows disease progression. When mice are treated at an early time point, before the development of a

palpable tumour, a striking difference in length of disease progression is observed between castrated and antiandrogen treated mice.

Reportable Outcomes

Data generated thus far has been presented locally at lab meetings and urology journal club and we have consulted with various members of the SPORE here at the University of Michigan and I believe that the project has evolved accordingly. The utilisation of the hAR mouse X TRAMP mouse model offers a unique advantage for analysis of antiandrogen mechanisms of action in vivo. This model provides the ability to study a 'humanised' androgen receptor in a transgenic model that has been shown to share similarities with human prostate cancer, including epithelial origin, progression from the PIN stage to adenocarcinoma, and metastasis by a transgene that is hormonally regulated by androgens.

Conclusions

Data obtained during this first year indicates that the xenograft models of prostate tumour growth produced in this study may not be the most appropriate tool to assess potential hormonal selection pressure on the production of AR variants. As discussed above we have proposed to utilise the hAR mouse x TRAMP model and I am confident that further study will highlight modes of AR action that will provide an invaluable insight into the mechanisms of androgen resistance.

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Zegarra-Moro OL, SchmidtLJ, Huang H, Tindal DJ. 2002, Disruption of androgen receptor function inhibits proliferation of androgen refractory prostate cancer cells. *Can. Res.* 62(4) 1008

Appendix

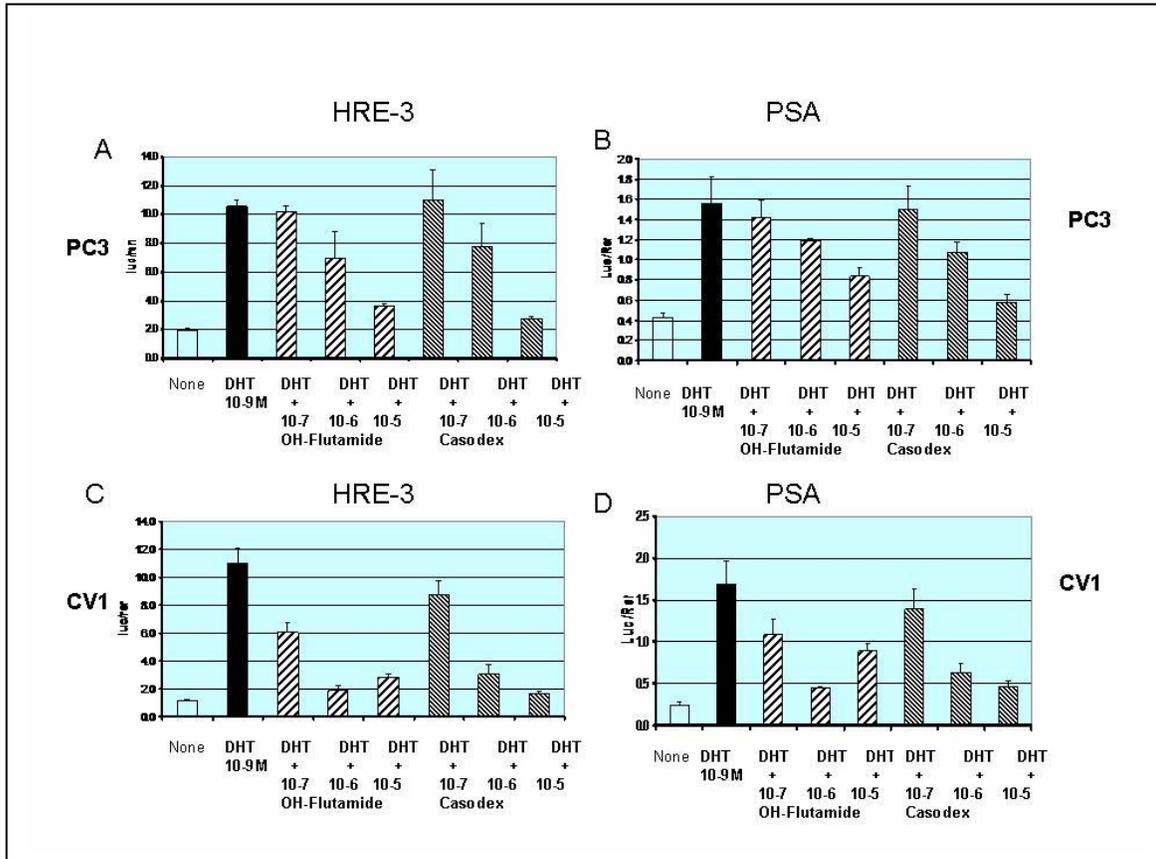


Fig 1 Flutamide agonist action is observed in CV-1 cells on two different promoters at high concentration. No agonist effect was observed in PC-3. 10^{-5} PC3 or CV1 cells were seeded into 24 well dishes and allowed to adhere for 24 hrs prior to transfection with 4 ng human androgen receptor and 20 ng renilla as a transfection control. Two reporters were utilised 200 ng 3xHRE luciferase reporter or 200ng PSA luciferase reporter.

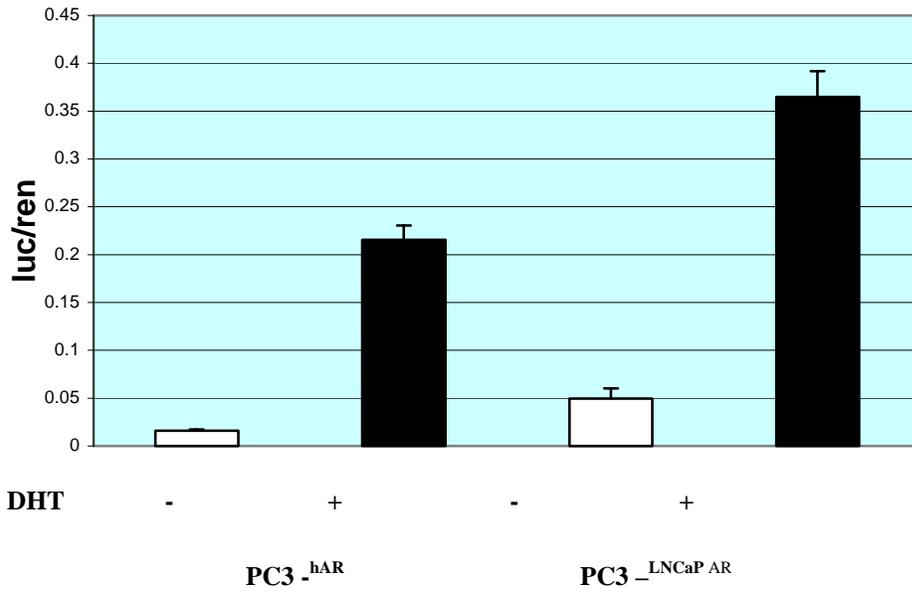
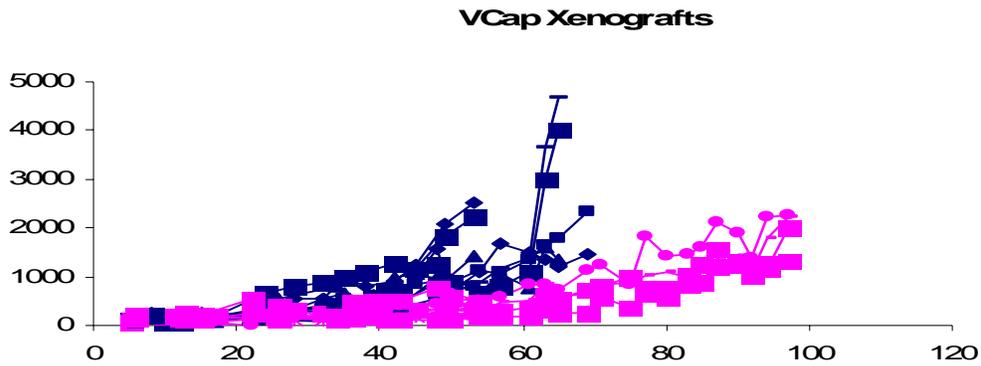


Fig 2 PC3-hAR and PC3-LNCaP AR cells were transfected with an androgen specific reporter (C'Δ9 tkta Luciferase, 1μg and CMV- Renilla, 10 ng as transfection control). Twenty-four hours after transfection cells were stimulated for a period of 24hr with 3×10^{-9} M DHT and harvested for analysis. Data is expressed as the ratio of firefly luciferase light units over corresponding renilla. (mean± SE).

A



B



C

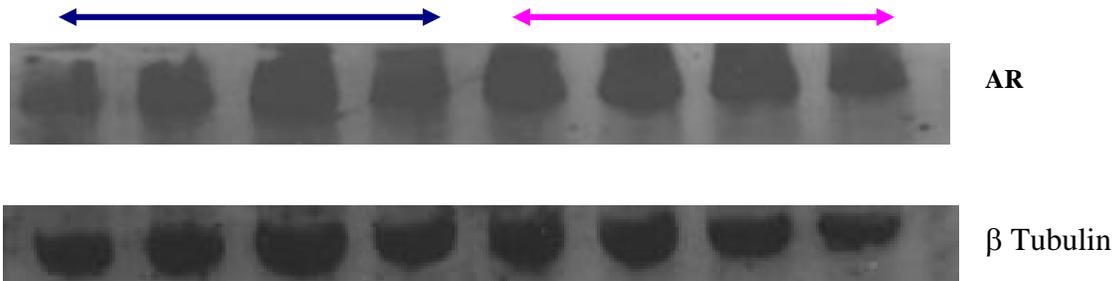


Fig 3 **A** Growth curve of VCaP xenograft tumours in intact (blue line) and castrate (Pink Lines) SCID mice. 3 million VCaP cells in PBS (150µl, 1:1 ratio to Matrigel) were implanted in the flank of each mouse and one week later 8 mice were castrated and 8 were not. **B** Immunostaining for human androgen receptor with AR -N20 (1:1000 overnight 4°C Santa Cruz). Positive staining is indicated by a brown precipitate. AR staining is predominately nuclear in both intact and castrated mice. **C**- Immunoblotting of total protein samples (50 µg/ lane) isolated from intact and castrate VCaP xenografted tumours (1:1000 overnight 4°C). β Tubulin (1:2000) was used as loading control.

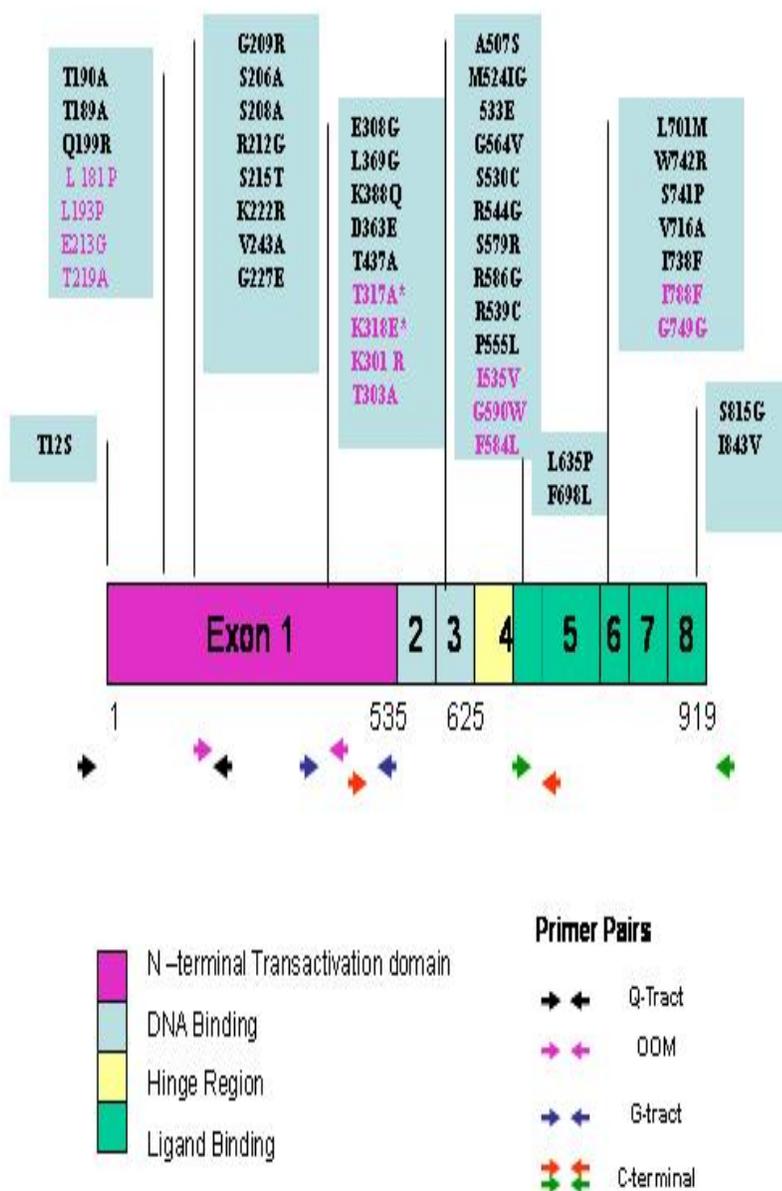


Fig 4 Graphical representation of the human androgen receptor with the location of primers utilised in amplification of the AR fragments. Mutations in black were identified in AR sequenced amplified from cDNA isolated from tumours from intact mice and those in pink text from castrated mice. Mutations identified remain to be verified by a second round of RT-PCR, sub-cloning and sequencing. Work is ongoing.

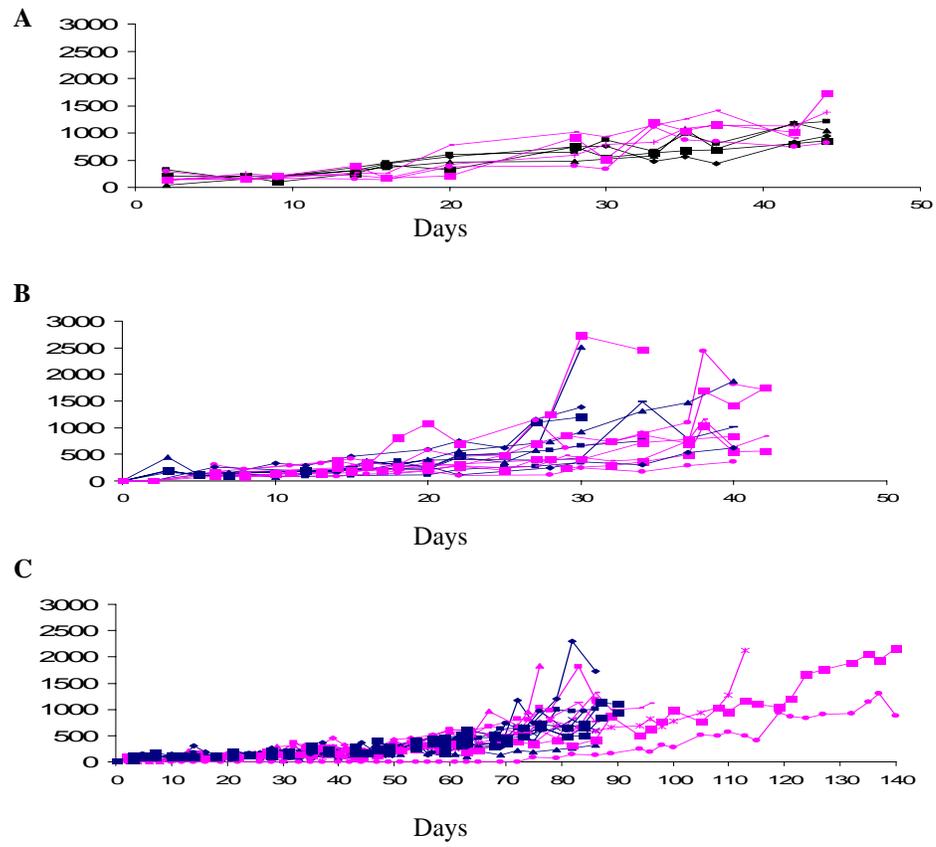


Fig 5 Graphs illustrating tumour volume increase over time in PC3 (A)-, -PC3-hAR (B), and PC3^{LNCAP AR} (C) tumour xenografts. 3 million cells in sterile PBS (1:1 ratio with Matrigel) were implanted s.c. into the flank of SCID mice and one week later eight of the mice were castrated. Navy lines represent intact SCID mice and pink lines represent castrated SCID mice.

A

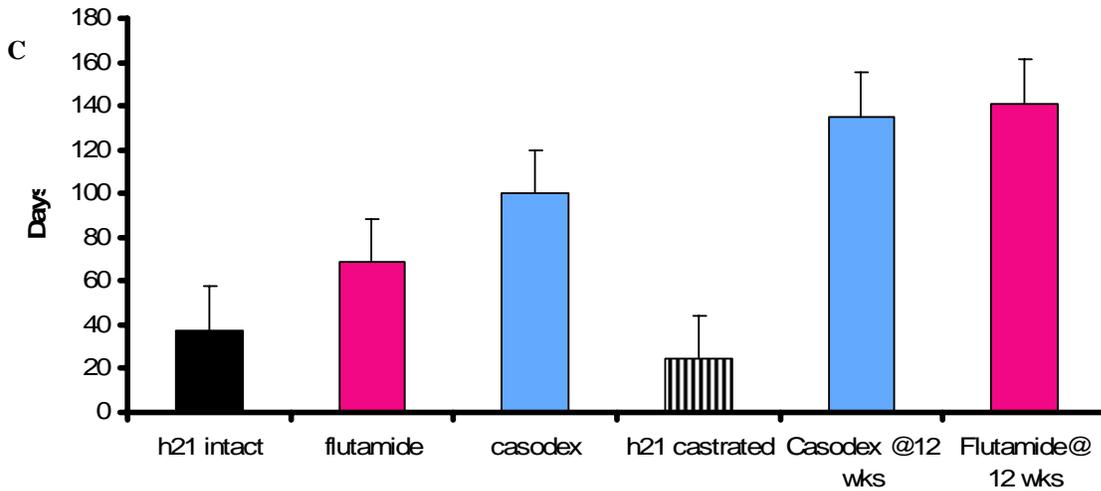
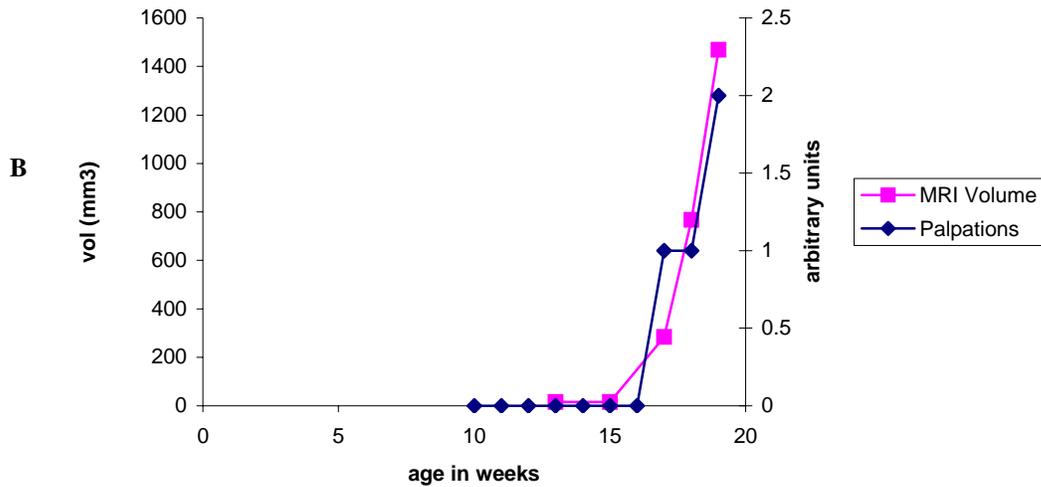
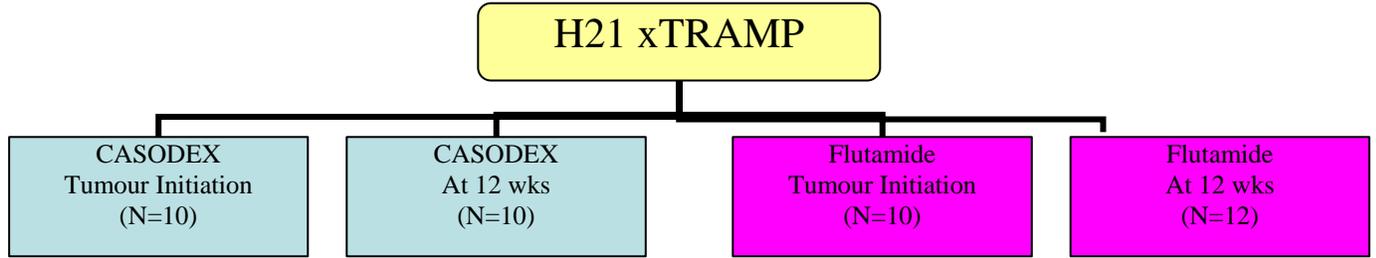


Fig 6 **A** Graphical representation of experimental strategy. **B** Comparison of palpation detection (arbitrary scale 1, 2, 3) and MRI measurement of a prostate tumour found in TRAMP x hAR mice. **C** Length of disease from first palpable tumour in hARx TRAMP mice to death.