EXPERIMENTAL STUDY

A yeast-based functional assay for the detection of the mutant androgen receptor in prostate cancer

Jocelyn Céraline¹, Eva Erdmann¹, Philippe Erbs², Marion Deslandres-Cruchant¹, Didier Jacqmin³, Brigitte Duclos⁴, Claudine Klein-Soyer¹, Patrick Dufour¹,⁴ and Jean-Pierre Bergerat¹,⁴

¹Laboratoire de Cancérologie Expérimentale et de Radiobiologie, EA 3430-ULP, IRCAD, 1, Place de l’Hôpital, BP426, F67091 Strasbourg, France,
²Transgene SA, F67000 Strasbourg, France, ³Department of Urologic Surgery, CHRU, 1, Place de l’Hôpital, BP426, F67091 Strasbourg, France, and
⁴Department of Haematology and Oncology, CHRU, F67100 Strasbourg, France

(Correspondence should be addressed to Jocelyn Céraline; Email: jocelyn.ceraline@ircad.u-strasbg.fr)

Abstract

Objective: Mutations in the ligand-binding domain of the human androgen receptor (AR) figure among the ways used by prostate adenocarcinoma (PCa) cells to escape androgen dependence. These mutations may broaden the specificity and/or affinity of the AR to other hormones, resulting in inappropriate receptor activation and thus affecting the PCa response to physiological stimuli and hormonal therapies.

Design: In order to clarify the impact of these mutations on disease progression and treatment, we have developed a yeast-based functional assay that allows the detection of mutant ARs and the analysis of their transactivation capacities in response to different ligands.

Methods: AR cDNA was directly cloned into an expression vector in a yeast strain that carries a reporter gene (ADE2) linked to an androgen-dependent promoter. The expression of the ADE2 gene and consequently the yeast cell growth in a selective medium depleted in adenine depends on the specificity of the AR for the ligand added to the medium.

Results: By analysing the transactivation capacities of different AR molecules in response to a broad range of steroid and non-steroid ligands, we have demonstrated that this assay can discriminate among wild-type AR, T877A, C685Y and L701H mutant ARs and that at least 1% of mutant ARs could be detected when mutant and wild-type ARs were mixed at the cDNA level.

Conclusions: The data presented here show that this simple AR assay is convenient for the routine detection of mutant ARs in PCa and is also suitable to evaluate the antagonist activities of anti-androgen molecules.

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Introduction

Prostate adenocarcinoma (PCa) is the most frequently diagnosed malignancy in men and the second leading cause of cancer death in western countries. Androgens play a critical role in controlling the growth, differentiation and function of the normal prostate by interacting with the androgen receptor (AR), a ligand-dependent transcription factor (1). Decreasing the plasma testosterone concentration by medical or surgical castration, eventually combined with anti-androgens, is the standard first-line therapy for advanced PCa. The response to such androgen ablation is high and provides relief to more than 80% of patients (2). Unfortunately, the efficacy of this therapy is only transitory, lasting some months or years, and all PCas will eventually relapse after a median duration of 12–18 months to reach an ‘androgen-independent’ status. Androgen-refractory cells still require the AR for proliferation, even in the absence of androgens (3). Different mechanisms have been proposed to explain the androgen independence, including activation of the AR in a ligand-independent manner through cross-talk with the human epidermal growth factor receptor-2 (HER2) and mitogen-activated protein kinase (MAPK) phosphorylation signal cascade (4–7), the overexpression of AR co-activators (8, 9) and mutations in the ligand-binding domain (LBD) of the receptor that expand its specificity and/or affinity to other hormones (10–14). Some authors have reported a relatively high frequency of mutations in metastatic PCa escaping androgen deprivation (12). The first mutation reported to lead to androgen independence was a missense mutation that caused a substitution of alanine for threonine at amino acid 877 (T877A) (15). This mutant AR is responsive to oestrogens and...
progestagens, as well as to anti-androgens such as flutamide. This mutation is frequently detected in patients with metastatic PCa who received combined androgen blockade with flutamide as an anti-androgen (16–18). Since then, additional mutations have been described, including the V715M mutant AR which binds adrenal androgens with higher affinity than wild-type AR (19), the C685Y mutant which binds oestrogens, progesterone and anti-androgens (20), and the L701H and L701H/T877A mutants which are responsive to glucocorticoids (21, 22). In advanced PCa, the presence of tumour clones with such AR mutations should be taken into consideration as hormonal therapies may ultimately exert a positive selection of these tumour cells. Therefore, the detection and characterization of these mutations are important to improve the endocrine therapy and to develop new anti-hormones directed against these subsets of androgen-independent clones in metastatic PCa. We have developed a yeast-based assay that allows both the detection of mutant ARs and the analysis of their transactivation capacities in response to different ligands. This assay is derived from the functional analysis of separated alleles in metastatic PCa. We have developed a yeast-based assay that allows both the detection of mutant ARs and the analysis of their transactivation capacities in response to different ligands. This assay is derived from the functional analysis of separated alleles in metastatic PCa. We have developed a yeast-based assay that allows both the detection of mutant ARs and the analysis of their transactivation capacities induced by a mutation that can be extraplated to the PCa subclone carrying this mutation. We first used this method to analyse the responsiveness of previously described mutant ARs to different native and non-classical ligands and suggest that this yeast-based assay is simple, sensitive and convenient for the routine detection and analysis of mutant ARs in PCa.

Materials and methods

Materials

D-aldoosterone, androstenedione, β-oestradiol, cortisol, cortisone acetate, dehydroepiandrosterone (DHEA), DHEA-sulphate (DHEA-S), dihydrotestosterone (DHT), flutamide, medroxypregosterone, pregnenolone, progesterone, testosterone, adrenaline, β-glucose, cytosine, uracil and all amino acids were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Nilutamide was a gift from Dr C Sultan (INSERM U439, Montpellier, France).

Construction of plasmids

Wild-type human AR (ARWT) cDNA (Genbank: NM_000044) has been amplified from plasmid pAR0 (Dr A O Brinkmann, Erasmus University, Rotterdam, The Netherlands) by PCR using primers p1 (5'-TCAGCTGTCATAATGGA-3', nt 1099-1119) and p2 (5'-CAGAGGAGTACTCGAGGATTA-3', nt 3959-3939). The italicized sequences refer to the created SpeI and XhoI restriction sites respectively in p1 and p2. The start codon is in bold characters. Primer p1 has been engineered to provide optimum translation initiation in yeast (26). The amplified SpeI-XhoI AR fragment was then cloned into plasmid pL242GFP (27). Thereafter, the 3’ untranslated sequence of AR was elongated by inserting into the XhoI site at position 3949 a fragment corresponding to positions 3938-4190 yielding the AR expression vector pARWT. This fragment was amplified by PCR from human prostate cDNA (Clontech Laboratories, Ozyme, Saint Quentin Yvelines, France) using primers p3 (5’-ATAACTCTCAGTACTCCCTCTGCA-3’, nt 3938-3961) and p4 (5’-TGACACTCTGAGGCCATATGGGATG-3’, nt 4170-4191). The ARWT sequence was checked thereafter by sequencing.

The gap repair vector pARΔ was derived from pARWT by deleting the BstEII-EcoRI fragment encompassing positions 2382 and 4015 of AR. Both pARΔ and pARWT are high copy number yeast episomes with the advantage that the AR gene, located on chromosome X, is monoallelic in men. Thus, any changes in AR transactivation capacities induced by a mutation can be extrapolated to the PCa subclone carrying this mutation. We first used this method to analyse the responsiveness of previously described mutant ARs to different native and non-classical ligands and suggest that this yeast-based assay is simple, sensitive and convenient for the routine detection and analysis of mutant ARs in PCa.

Total RNA isolation and reverse transcription

Total RNA were prepared from 10⁶ LNCaP cells with Trizol reagent (Invitrogen Life Technologies) and
quantified by spectrophotometry (Amersham Pharmacia Biotech, Saclay, France). For cDNA synthesis, 2 μg total RNA from LNCaP or from human prostate (Clontech Laboratories) were mixed with 500 ng oligo d(T)12–18 (Invitrogen Life Technologies) in 12 μl denatured for 5 min at 65°C and chilled. The reverse transcription was then performed in the presence of 0.5 mmol/l dATP, 0.5 mmol/l dCTP, 0.5 mmol/l dGTP, 0.5 mmol/l dTTP, 50 mmol/l Tris–HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl2, 10 mmol/l dithiothreitol (DTT), 2 U/μl RNaseOUT recombinant ribonuclease inhibitor and 200 U/μl RNase H− reverse transcriptase (Invitrogen Life Technologies) for 1 h at 42°C. The reaction was terminated by heating for 15 min at 70°C.

**Generation of AR mutants**

The mutant T877A was derived from the LNCaP cell line by RT-PCR using forward primer 5′-TGCGGGCGGGCAGTGGCGCTAT-3′ and reverse primer 5′-GTTGCGCATGGAGGTTAGGAGGAG-3′. The amplified product was thereafter digested with BstEII and XhoI and subsequently exchanged with the corresponding pAR WT fragment, yielding pAR T877A. Mutations C685Y and L701H were created in pAR WT by ‘overlapping’ PCR (31). Final constructs named pAR T877A, pAR L701H and pAR C685Y were checked by sequencing.

**Strains, media and general techniques**

The Saccharomyces cerevisiae strain YPH250 (MATa ade2-101 o his3-D200 leu2-Δ1 lys2-801 Δ trp1-Δ1 ura3-52) (28) was used as parent strain. The pRS/ARE-Ade2 construct was linearized with NcoI within the URA3 marker and integrated into the ura3 locus of YPH250 to yield the androgen-dependent strain EJ250 (MATa ade2-101 o his3-D200 leu2-Δ1 lys2-801 Δ trp1-Δ1 ura3-52 URA3::ARE-ADE2 (pRS/ARE-Ade2)). Strains EJ250, EJ250 C685Y, EJ250 L701H and pAR T877A were derived from EJ250 by the introduction of plasmid pAR WT, pAR C685Y, pAR L701H and pAR T877A respectively. Strain EJ250 was derived from EJ250 by introducing the empty p424GFP plasmid and was used as the negative control in our assay. Standard yeast manipulations were performed as described in Yeast Protocols Handbook (Clontech Laboratories, Inc., CA, USA) and in Balamelli–Gallacchi et al. (25). The selective medium for the ADE2 system was depleted in adenine, tryptophan and uracil and consisted of 2% d-glucose, 0.67% yeast nitrogen base (DIFCO Laboratories, Detroit, MI, USA), 2% agar, and all amino acids except tryptophan. For the screening of AR responsiveness to ligands, the medium was specifically supplemented with the adequate steroid or anti-androgen.

**Primers and PCR conditions**

An AR fragment (1786 bp, nt 2311–4097) was amplified by PCR from the different AR expressing plasmids or from human prostate cDNA with the forward primer AR1 (5′-TGCGGGCGGGCAGTGGCGCTAT-3′) and the reverse primer AR2 (5′-GTTGCGCATGGAGGTTAGGAGGAG-3′). This fragment encompassed the DNA binding domain, the LBD and the AF-2 domain of AR. The PCR reaction was performed in the presence of 1.25 U Platinum Pfx DNA polymerase (Invitrogen Life Technologies), 2 × PCRx enhancer solution (Invitrogen Life Technologies), 0.3 mmol/l of each dNTP, 1 mmol/l MgSO4 and 0.3 μmol/l of each primer. The PCR consisted of 30 cycles (45 s at 94°C, 45 s at 55°C, 2.5 min at 68°C) and 5 min at 68°C. The amplified fragments were then purified (PCR purification kit; Qiagen SA, Courtaboeuf, France) and used for the gap repair assay.

**Gap repair assay**

Aliquots of 50 μl yeast (2 × 10⁹ cells/ml) were co-transformed by the LiAc/PEG method (32, 33) with 100 ng linearized pAR Δ gap repair vector. 100 ng purified AR PCR fragment and 100 μg heat-denatured salmon sperm DNA. After transformation, the yeast was pelleted and resuspended in 400 μl sterile deionized water and 100 μl were spread on selective medium supplemented with the appropriate hormone or anti-androgen. After incubation for 72 h at 30°C, colonies were scored. With each set of transformations a negative control, the linearized gap repair plasmid alone, was included.

**Western blot analysis**

Two OD₆₀₀ₐ₅ᵓ units of an exponential yeast culture were transferred into a 13 mm diameter tube containing 2 ml 50 mmol/l Tris (pH 7.5) and 10 mmol/l NaNO₃ on ice and centrifuged. Cells were lysed with 150–212 μm acid-washed glass beads (Sigma–Aldrich) in 30 μl lysis buffer (2% SDS, 80 mmol/l Tris (pH 6.8), 10% glycerol, 1.5% DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mmol/l phenylmethyl sulfonyl fluoride (PMSE) and 0.1 mg/ml bromophenol blue) by vortexing on high four times for 45 s with 30 s on ice between each mixing. Thereafter, 70 μl lysis buffer were added and samples were heated to 100°C for 1 min. Extracts of 30 × 10⁶ LNCaP cells were prepared in lysis buffer (100 mmol/l Tris–HCl (pH 7), 140 mmol/l NaCl, 3 mmol/l MgCl₂, 0.5% Nonidet-40, 2 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). Cellular debris was removed from lysates by centrifugation at 14 000 g and protein extracts were boiled for 5 min in Laemmli buffer, loaded onto 10% SDS gel and transferred to nitrocellulose membranes by electroblotting. Blots
were probed with Ab-3, a rabbit IgG polyclonal antibody against human AR (Oncogene Research Products, Merck Eurolab, Fontenay-Sous-bois France) and peroxidase-conjugated secondary antibodies (sheep anti-rabbit IgG, Amersham Pharmacia Biotech, France). Detection was performed with the chemiluminescence Western blotting kit (Amersham Pharmacia Biotech).

**Results**

**Construction of an androgen-inducible expression system**

We have constructed a *S. cerevisiae* strain in which the expression of the ADE2 gene coding for an enzyme that intervenes in the adenine biosynthesis pathway is

**Figure 1** Functional assay of AR in yeast. The expression of the ADE2 gene is androgen dependent. (A) Activation of the AR<sub>WT</sub> by DHT or testosterone (T) leads to the expression of the ADE2 gene resulting in yeast growth on selective medium lacking adenine. (B) There is no expression of the ADE2 gene and therefore no yeast growth in the presence of steroid ligands that AR<sub>WT</sub> binds to with low affinity. (C) Mutant (Mut) AR with a broad specificity can be activated by different ligands resulting in yeast growth on selective medium lacking adenine. p, plasmid.

**Figure 2** Western blot of AR from different sources. Aliquots of protein extract from LNCaP cells, the parental yeast strain EJ250, EJ25<sub>424GPD</sub>, EJ25<sub>WT</sub>, EJ25<sub>T877A</sub>, EJ25<sub>C885Y</sub> and EJ25<sub>L701H</sub> were separated on 10% SDS-PAGE. Blots were revealed with a polyclonal antibody against AR (110 kDa) (Ab-3; Oncogene Research).
induced by the human AR. This yeast strain contains an integrated plasmid with the ADE2 open reading frame under the control of a URA3 gene minimal promoter (29) in which the sequences required for both basal and activated transcription have been replaced with three AREs. When the strain is transformed with a plasmid encoding AR WT and plated on medium lacking adenine and containing either a testicular androgen, testosterone (T) or DHT or an adrenal androgen, androstenedione, DHEA or DHEA-S at the concentrations indicated. Activation of the ADE2 gene leads to yeast growth.

**Gap repair assay**

Total RNA isolated from LNCaP cells or commercially available total RNA from human prostate tissue was reverse transcribed. A 1786 nt AR fragment (nt 2311-4097) was amplified by PCR from the obtained cDNA and purified. The purified AR PCR product was transformed into yeast cells together with the linearized pARΔ gap repair vector. Since the gap repair vector was linearized between positions 2382 and 4015 of AR WT, the entire DNA-binding, ligand-binding and AF-2 domains of AR were tested. The AR fragment was integrated into the expression vector by homologous recombination resulting in expression of the AR in yeast cells.

**Expression of AR in yeast and androgen-dependent activation of ADE2 gene**

Expression of AR in S. cerevisiae was assayed by Western blot. AR was expressed in our yeast strains and was indistinguishable from the AR expressed in human cells. Figure 2 shows the results of a Western blot performed on cytosol extracts of yeast strains EJ25 WT, EJ25424GPD, EJ25 WT, EJ25 C685Y, EJ25 L701H and EJ25 T877A and of the prostate cancer cell line LNCaP. For EJ25 WT, EJ25 C685Y, EJ25 L701H and EJ25 T877A, an immunoreactive protein band with an apparent molecular weight of 110 kDa, similar in size to the AR expressed in the LNCaP cell line, was observed. This band was not visible in host cells alone or in yeast strain EJ25 424GPD containing the empty p424 GPD vector.

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![Figure 3 Androgen-dependent activation of the ADE2 reporter gene.](image)

**Figure 3** Androgen-dependent activation of the ADE2 reporter gene. EJ25 WT yeast strain expressing the AR WT was plated on medium lacking adenine and containing either a testicular androgen, testosterone (T) or DHT or an adrenal androgen, androstenedione, DHEA or DHEA-S at the concentrations indicated. Activation of the ADE2 gene leads to yeast growth.

![Figure 4 Growth responses of EJ25 WT, EJ25 C685Y, EJ25 L701H and EJ25 T877A yeast strains to DHT, progesterone (Prog) and cortisol.](image)

**Figure 4** Growth responses of EJ25 WT, EJ25 C685Y, EJ25 L701H and EJ25 T877A yeast strains to DHT, progesterone (Prog) and cortisol. After transformation with the appropriate AR expression plasmid, the yeast strains were plated on selective media with 10 nmol/l DHT or 100 nmol/l progesterone or 100 nmol/l cortisol. EJ25 424GPD yeast strain served as negative control.
The ability of the AR<sub>WT</sub> to transactivate the ADE2 gene was thereafter determined by cell growth on selective media. In the experiment shown in Fig. 3, the EJ25<sub>WT</sub> yeast strain was plated on medium lacking adenine and containing either testosterone or DHT or one of the following adrenal androgens: androstenedione, DHEA or DHEA-S at the indicated concentrations. Yeast growth was observed on plates containing DHT or testosterone at concentrations as low as 10 nmol/l. No growth was observed without any ligand. As predicted from earlier studies (18), the response to adrenal androgens was very low. EJ25<sub>WT</sub> responded only to androstenedione and the minimum concentration required for growth stimulation was 1 μmol/l. Neither the DHEA nor the DHEA-S could stimulate the growth of EJ25<sub>WT</sub> in our model within the range of concentrations used. No growth was observed in these conditions for the AR negative control EJ25<sub>L701H</sub> strain (data not shown).

Transactivation capacities of wild-type and defined mutant ARs

To demonstrate that our yeast model is convenient for distinguishing between wild-type and mutant ARs, the yeast strain EJ250 was transformed with one of the AR expression plasmids and was plated on selective media with DHT, progesterone or cortisol. Figure 4 represents the growth responses obtained. The negative control, the yeast strain EJ25<sub>WT</sub>424GPD did not show any growth stimulation in response to the ligands tested. Yeast strains EJ25<sub>WT</sub>, EJ25<sub>C685Y</sub> and EJ25<sub>T877A</sub> responded similarly to 10 nM DHT. C685Y and T877A mutants were also responsive to 100 nmol/l progesterone. As predicted from earlier studies (22), the L701H mutant responded to a lesser extent to DHT and progesterone and had a substantial transactivation response to cortisol.

The responsiveness of wild-type and mutant ARs to different concentrations of a broad range of steroid and non-steroid ligands was further evaluated in order to establish hormonograms for each AR molecule. Figure 5 summarizes the results obtained with the AR<sub>WT</sub> and the T877A and C685Y mutants. The T877 mutant was less responsive to 10 nmol/l DHT than the AR<sub>WT</sub> and C685Y mutant. The response of both T877A and C685Y mutants to androstenedione was much greater than that of the AR<sub>WT</sub>. Both C685Y and T877A mutants were responsive to DHEA, flutamide, progesterone and medroxyprogesterone. None of the three receptors tested was activated by glucocorticoids (cortisone acetate, cortisol) or by the mineralocorticoid D-aldosterone. The AR<sub>WT</sub> was activated by the anti-mineralocorticoid spironolactone and this response was not altered by T877A and C685Y mutations.

**Figure 5** Dose–response histograms of wild-type AR, and T877A and C685Y mutants to different concentrations of a panel of steroid and non-steroid ligands, DHT, androstenedione (A), DHEA, flutamide (F), β-oestradiol (E2), progesterone (P), medroxyprogesterone (MP), cortisone acetate (CA), cortisol (C), aldosterone (Ald) and spironolactone (SP). Yeast was co-transformed with the corresponding AR PCR fragment and the linearized gap repair plasmid and plated on selective media supplemented with 10 nmol/l (open bars), 100 nmol/l (shaded bars), 1 μmol/l (stippled bars), 10 μmol/l (solid bars) or 100 μmol/l (hatched bars) of the appropriate ligand. For the negative control, yeast strains were plated without any ligand. Arrows indicate the mutation-induced changes in the specificity or affinity of the AR for some ligands. Data represent one experiment performed three times.

**Steroid and non-steroid ligand antagonist activity**

We have also evaluated in our system the antagonist activity of well-known anti-androgens. The non-steroidal anti-androgens flutamide and nilutamid and the steroidal anti-androgen cyproterone acetate were tested by incubating the yeast strain EJ25<sub>WT</sub> with 10 nmol/l DHT in the absence (100% proliferation in Fig. 7) or the presence of 100 μmol/l anti-androgen. The DHT-induced proliferation of the yeast strain EJ25<sub>WT</sub> decreased in the presence of flutamide and nilutamid to a value that accounted for 4 and 40% of the maximum DHT response respectively. These results indicated that the antagonist properties of different steroids can be tested in our model. In the presence of cyproterone acetate, the DHT-induced proliferation of the yeast strain EJ25<sub>WT</sub> was 98% of that observed with DHT alone. These results are in good agreement with the partial antagonist activity of cyproterone acetate.

**Determination of the sensitivity of the AR yeast-based functional assay**

As our goal was to detect mutant ARs between the wild-type AR expressed in metastatic PCa we determined the threshold beyond which a mutant AR could be detected with our assay. The EJ250 yeast strain was co-transformed with the gap repair vector and 100 ng wild-type and T877A AR PCR fragments mixed at different ratios. After transformation, yeast cells were plated on medium containing 100 nmol/l progesterone for the selective growth of colonies expressing the T877A mutant AR only. As illustrated in Fig. 6 a significant number of colonies was obtained with 1% of T877A mutant AR. This suggests that at least 1% of mutant AR can be detected in our assay when wild-type and mutant T877A were mixed at the cDNA levels.
Discussion

The presence of mutations in the LBD of the AR is one of the mechanisms proposed to explain how advanced PCa may escape androgen deprivation. These mutations expand the specificity and/or affinity of the AR to other hormones, resulting in inappropriate receptor activation by oestrogens, progestagens, adrenal androgens, corticoids and anti-androgens. A major challenge for the treatment of PCa would be to detect and analyse the transactivation capacities of these potential AR mutants in subsets of androgen-independent clones.

We describe here a convenient, simple and rapid yeast-based functional assay to selectively detect and characterize mutant AR in PCa. We have constructed a yeast strain in which the expression of the ADE2 gene is induced by the AR. We have demonstrated that although the yeast S. cerevisiae does not possess endogenous nuclear receptors (NRs), AR and several other NRs can function as ligand-dependent transactivators in yeast (34-39). It has also been shown that the transactivation functions (AF-1 and AF-2) of NRs in yeast are in many respects similar to those in mammalian cells (40).

The present assay has the advantage of being based on yeast proliferation instead of colony colour screening as previously described (24, 25, 41). Thus, only the yeast cells in which the AR is activated by the ligand added to the selective medium will proliferate and form colonies as shown for the T877A mutant in Fig. 6. Mutant ARs have a different affinity spectrum for steroid ligands compared with wild-type AR. In this assay, the yeast growth depends on the specificity of the AR expressed in the yeast strain for the steroid ligand present in the medium. Mutant ARs can therefore be distinguished from wild-type AR by comparing their specificity for a range of steroid hormones and anti-androgens.

We first checked for the expression of the AR in our yeast model by Western blotting and showed that both wild-type and mutant ARs were expressed in yeast and that the signal corresponded to the AR protein from LNCaP cell extracts. We have demonstrated that, when expressed in yeast, AR was able to transactivate the ADE2 reporter gene and that this function was strictly hormone dependent.

Subsequently, we have evaluated the specificity of the wild-type AR expressed in yeast for a range of steroid
ligands including DHT, testosterone and adrenal androgens. In our assay, the lowest concentration of DHT required for the yeast growth was 10 nM, which is similar to the concentration required in mammalian systems for maximal activity (22). The response of wild-type AR to the different steroids tested was as predicted with a decreasing affinity for DHT, testosterone, androstenedione, DHEA and DHEA-S (14, 42, 43). The assay was further tested with different mutant ARs, and the results obtained show that ARL701H, ART877A and ARC685Y present a different profile of activation by the ligands tested compared with ARWT. The L701H mutant was, as expected, the only one to be responsive to the glucocorticoid cortisol. Interestingly, ART877A exhibited the highest response to progestagens and was also the only one that was activated by micromolar concentrations of pregnenolone, the precursor of progesterone (data not shown). Surprisingly, both ART877A and ARC685Y responded to nanomolar concentrations of the adrenal androgen androstenedione. Accordingly, these two mutants were also more responsive to medroxyprogesterone, a synthetic derivative of 17α-hydroxyprogesterone which is the precursor of androstenedione. Moreover, the testing of antagonist activities of anti-androgens was possible with our assay. The non-steroidal anti-androgens flutamide and nilutamide were able to inhibit the androgen-dependent growth of yeast cells expressing the wild-type AR. Together, these data suggest that when this assay is applied to analyse the responsiveness of AR to a panel of steroid ligands, mutant ARs can be distinguished from the wild-type receptor. Furthermore, this assay will be useful to design the most efficient antagonist molecule for a particular mutant receptor.

The first application of this assay will be to detect new AR mutations that modify the transactivation capacities of the AR. By performing the assay on total RNA isolated from biopsies of the primary tumour and then from hormone-sensitive metastases and ultimately from hormone-resistant metastatic foci, we should be able to detect the clonal outgrowth of androgen-independent cells from the initial tumour during the hormonal therapy. Indeed, it is established that the development of androgen resistance is due to the emergence of androgen-independent cancer cells from the tumour (44, 45) and Taplin and colleagues (18) have demonstrated that the AR mutation T877A occurs preferentially in PCas treated with the anti-androgen flutamide. Thus, detecting very early androgen-independent cells with mutant AR in primary tumours could have significant implications for the treatment of PCa and the assay we have developed will be useful for this purpose. However, it remains to be ascertained whether this assay is sensitive enough to detect these androgen-independent cells that account for 1 in 10^7–10^8 cells in the initial tumour as Craft and colleagues (44) have postulated. Nevertheless, we have demonstrated that at least 1% of mutant AR could be detected when mutant and wild-type ARs were mixed at the cDNA levels.

Finally, data recovered from this assay will provide better information about the natural history of PCa disease. The results obtained will suggest the most suitable hormonal therapy for hormone-refractory metastatic PCa in which specific agonists or antagonists will be recommended or advised against on a patient-by-patient basis. For instance, it is known that androgen ablation causes more than a 90% reduction in plasma testosterone level, but only a slight decrease in plasma adrenal androgen levels (46) and the results obtained with mutants ART877A and ARC685Y show that they were both responsive to androstenedione, an adrenal androgen with which wild-type AR binds with low affinity. Thus blocking the activity of the remaining androstenedione or, even more, reducing its plasma level with glucocorticoids may be worth considering as a more appropriate treatment of PCas that express such mutant ARs.

In conclusion, we have demonstrated that the AR assay presented here can be used to detect AR gene mutations in PCa by analysing the responsiveness of the AR mutant to a battery of steroid and non-steroid ligands. This assay, which is sensitive, rapid and inexpensive, will provide interesting data on the transactivation capacities of the mutant AR detected. In addition, since the efficiency of antagonist molecules can also be assessed, this assay will be useful to improve clinical data in order to ameliorate the treatment of PCa with a particular mutant AR.

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