INTRODUCTION

Androgen deprivation therapy (ADT), as a standard treatment for metastatic prostate cancer (PCa), is initially effective in reducing tumor burden, palliating symptoms and improving overall survival. However, patients will eventually experience disease progression and develop castration-resistant prostate cancer (CRPC) within 1–3 years. CRPC patients have a poor prognosis, with a median survival of ~30 months (1). On the basis of recent insights in the role of androgen receptor (AR) signaling in CRPC, several new therapeutic approaches are now being tested in CRPC (2). Abiraterone, a CYP17 inhibitor, was recently approved for chemotherapy-resistant CRPC, and a number of (other) drugs interfering with AR signaling are now being tested in a clinical trial. Until now, there is no clear way to individualize these new therapies. This step might be possible if we better understand the various mechanisms that lead to CRPC. Hence, an individualized treatment in CRPC on the basis of individual molecular tumor profiling is needed to raise the benefit of treatment. Furthermore, molecular tumor profiling may provide an opportunity to develop and implement novel innovative drugs (3,4).

Current endocrine treatment for advanced prostate cancer does not result in a complete ablation of adrenal androgens. Adrenal androgens can be metabolized by prostate cancer cells, which is one of the mechanisms associated with progression to castration-resistant prostate cancer (CRPC). Aldo-keto reductase family 1 member C3 (AKR1C3) is a steroidogenic enzyme that plays a crucial role in the conversion of adrenal androgen dehydroepiandrosterone (DHEA) into high-affinity ligands for the androgen receptor (testosterone (T) and dihydrotestosterone (DHT)). The aim of this study was to examine whether AKR1C3 could be used as a marker and therapeutic target for CRPC. AKR1C3 mRNA and protein levels were upregulated in CRPC tissue, compared with benign prostate and primary prostate cancer tissue. High AKR1C3 levels were found only in a subset of CRPC patients. AKR1C3 can be used as a biomarker for active intratumoral steroidogenesis and can be measured in biopsy or transurethral resection of the prostate specimens. DuCaP (a CRPC cell line that has high AKR1C3 expression levels) used and converted DHEA under hormone-depleted conditions into T and DHT. The DHEA-induced growth of DuCaP could be antagonized by indomethacin, an inhibitor of AKR1C3. This study indicates that AKR1C3 can be considered a therapeutic target in a subgroup of patients with high AKR1C3 expression.
prostatectomy. Primary PCa cases were selected from patients without any previous hormonal treatment. The specimens were snap-frozen in liquid nitrogen or formalin fixed and paraffin embedded. Tissues were processed by step sectioning, and the samples for analysis were selected after histopathological confirmation of the tissue to be CRPC, primary PCa, BPH or normal prostate.

Real-Time Polymerase Chain Reaction
TRIzol-extracted total RNA (2 μg) was treated with DNase I, and cDNA was synthesized by using random hexamer primers and SuperScript II-MMLV reverse transcriptase (Invitrogen; Life Technologies, Carlsbad, CA, USA). The reverse transcriptase (RT) reaction (30 μL) was diluted four times in H₂O (milliQ). Gene expression was determined by SYBR Green qPCR (quantitative polymerase chain reaction) by using SYBR Green PCR mix (Roche Diagnostics, Mannheim, Germany) and 2 μL cDNA as a template. RNA not subjected to reverse transcription was used as a negative control for PCR amplification. Gene-specific primers were used as described previously described (15). qPCR was performed on a LightCycler LC480 instrument (Roche Diagnostics) by using the following amplification conditions: 5 min 95°C, followed by 45 cycles of 10 s at 95°C, 20 s at 60°C and 20 s at 72°C. Crossing-point (Cp) values were determined by using LightCycler 480 SW 1.5 software (Roche Diagnostics). Hypoxanthine ribosyltransferase (HPRT) expression was used for normalization (16). Relative gene expression levels were calculated according to the model described by Pfaffl (17).

Western Blot Analysis
Total protein extracts were collected in Laemmlı lysis buffer (2% sodium dodecyl sulfate [SDS], 60 mmol/L glycine, pH 6.8, 1 mmol/L CaCl₂, and 300 mmol/L β-mercaptoethanol). Proteins were separated in 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), transferred to a polyvinylidene fluoride (PVDF) transfer membrane (Amersham Hybond-P; GE Healthcare Europe GmbH, Freiburg, Germany) and probed with a monoclonal mouse-anti-human AKR1C3 antibody (NP6.G6.A6, 1:1,000; Abcam, Cambridge, UK) and anti-β-actin (1:5,000; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) for loading control. The signal was detected by ECL Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer’s protocol. Protein expression levels were obtained after densitometric scanning of the ECL films on a GS-690 densitometer (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands).

Immunohistochemistry
Paraffin-embedded tissues were used for immunohistochemistry. Kidney and heart were used as AKR1C3-positive and -negative control tissues, respectively. The protocol was slightly modified from a previous study (18). Briefly, 5-μm tissue sections were cut, mounted onto Superfrost plus object slides and then baked at 60°C for 1 h. After deparaffination, antigen retrieval was performed with 10 mmol/L sodium citric acid buffer (pH 6.0) at 95°C for 1 h. Nonspecific binding was blocked by incubating the tissue sections in 0.1 mol/L Tris-HCl (pH 7.6) containing 10% rabbit serum for 2 h. Human AKR1C3 was detected by monoclonal mouse NP6G6.A6 antibody (Abcam) at a 1:1,000 dilution in the blocking buffer. Primary antibody was incubated overnight at 4°C in a moist chamber. Rabbit anti-mouse peroxidase secondary antibody was applied for 1 h at room temperature. DAB-H₂O₂ (ImmunoLogic BV, Duiven, the Netherlands) substrate was added to the slides and incubated at room temperature for an additional 4 min. Tissue sections were counterstained lightly with hematoxylin. Slides were dehydrated and sealed with Permount Mounting Media (SP15-500; Fisher Scientific, Hampton, NH, USA) for visualization by light microscopy.

Cell Culture
Prostate cell lines are described in SUPPLEMENTARY Table S1. All cells were cultured in RPMI-1640 medium (31870; Invitrogen;
Life Technologies), supplemented with l-glutamine and 10% fetal bovine serum (F7524; Sigma-Aldrich), without antibiotics, except LAPC4, Ep156T and DuCaP-N, which were cultured as described previously (19–21). Cells were maintained in a humidified atmosphere at 37°C and 5% CO₂. For DuCaP experiments, we added 100 U penicillin/mL and 100 μg streptomycin/mL (Invitrogen; Life Technologies) in the medium. The DuCaP passage numbers were 35–55.

**Proliferation Assay**

Cells were incubated in RPMI-1640 supplemented with 10% charcoal-stripped serum (CSS), supplemented with 1 nmol/L DHEA (D4000; Sigma-Aldrich), to resemble circulating adrenal androgen conditions in ADT and CRPC patients (5,22). Cells were seeded in 96-wells plates (Corning B.V. Life Sciences, Amsterdam, the Netherlands) at a density of 10,000 cells per well. Half of the medium was changed every forth day. A standard MTT (dimethylthiazolyldiphenyltetrazolium bromide; Sigma-Aldrich) assay was performed every forth day up to d 16; readings were performed on a Victor3 multimeter (PerkinElmer, Waltham, MA, USA). Experiments were performed in triplicate and were repeated at least three times. Relative cell numbers were calculated from optical density values.

**Radioimmunoassay**

Conditioned media were collected from the cells that were incubated in six-well plates (Corning) with 100,000 cells/well. AND, T and DHT were assessed from conditioned medium by 3H-radioimmunoassay after prepurification by using paper chromatography of ether extracts of the samples, including correction for procedural losses, as described previously (23–25). To summarize briefly, before extraction, 3H-AND, 3H-T or 3H-DHT was added to correct for procedural losses. After chromatography, the location of the AND, T or DHT zone was identified by radiochromatogram scanning, and the zone was cut out and soaked in buffer. The recovered radioactivity was measured by liquid scintillation counting of an aliquot from the eluate. Subsequently, AND, T or DHT tracer and antiserum were added, and after incubation, free and bound tracers were separated by means of dextrane-coated charcoal. Antibody-bound radioactivity was assessed by liquid scintillation counting of the supernatant. The calculations were performed by special software designed for correction of the mass and radioactive contribution of the recovery tracer in the radioimmunoassay. The detection limit for AND was 50 pmol/L when using a sample volume of 1 mL. The intra-assay coefficient of variation (CV) between-run was 7.6% at a level of 4.2 nmol/L. The detection limit for T was 16 pmol/L when using a sample volume of 1 mL. The CV between-run was 5.8% at a level of 6.5 nmol/L. Accuracy was assessed by measurement of quality-control samples in which the T levels had been assessed by gas chromatography–mass spectrometry. A bias of –1.3 ± 1.7% (standard error of the mean) compared with the target value was observed.

**Intervention Experiment**

Indomethacin (INN) (I7378; Sigma-Aldrich), an AKR1C3 inhibitor (26), was diluted in ethanol. INN concentrations of 10 and 25 μmol/L were added to DuCaP cell culture medium every 2 d. The control group was treated with vehicle (that is, ethanol equal to the amount of ethanol in the 25 μmol/L INN group). Proliferation and androgen levels were measured as explained above.

**Apoptosis Assay**

DuCaP cells (1 × 10⁶/well) were grown on clear-bottom black 96-well plates (Corning) for 24 h. Then the cells were treated with INN as mentioned above. Caspase-3/7 activity was measured at 2–48 h after treatment by using the Apo-ONE assay (Promega Benelux B.V., Leiden, the Netherlands) according to the manufacturer’s instructions. Experiments were done twice in triplicate.

**Cell Cycle Analysis**

DuCaP cells (2 × 10⁶) were grown and treated with INN for 2 d. Attached and floating cells were harvested in Hank’s Balanced Salt Solution (Invitrogen; Life Technologies) and fixed in 70% ethanol on ice. Samples were treated with 100 μg/mL Ribonuclease A (Sigma-Aldrich) for 40 min at 37°C, and DNA was stained with 40 μg/mL propidium iodide (Sigma-Aldrich). Cells (100,000) were analyzed on a FC500 flow cytometer (Beckman Coulter Nederland B.V., Woerden, the Netherlands). Experiments were performed two times.

**Statistical Analysis**

The correlation of AKR1C3 mRNA level by qPCR and protein level by Western blot was analyzed using linear regression. The effect of DHEA and INN treatment on cell proliferation and apoptosis was analyzed by using a one-way analysis of variance (ANOVA) test with post hoc Dunnett correction and two-way ANOVA tests with post hoc Bonferroni correction on relative cell numbers on d 0 compared with other time points. Androgen level was analyzed using a one-way ANOVA test with post hoc Dunnett correction. Graphpad Prism 5 software was used and p values <0.05 were considered statistically significant.

All supplementary materials are available online at www.molmed.org.

**RESULTS**

**AKR1C3 Is Overexpressed in a Subset of CRPC Cases**

A total of 15 BPH or nonmalignant prostate, 30 primary PCa and 44 CRPC samples were evaluated for AKR1C3 mRNA and protein expression levels. All normal and primary PCa samples had low AKR1C3 mRNA expression, whereas a subset of CRPC cases showed upregulation of AKR1C3 at the RNA level (Figure 1A). AKR1C3 mRNA expression was confirmed by Western blot analysis (Figure 1B). There was a correlation between
AKR1C3 mRNA and protein levels ($r^2 = 0.39$, $p < 0.0001$). Western blot analysis results were confirmed by immunohistochemistry. One (of 30) primary PCa cases displayed weak AKR1C3 immunoreactivity (Figure 1C, image 1). Strong AKR1C3 immunohistochemical staining was found in 34% (15 of 44) of CRPC cases (Figure 1C, image 2), and a moderate to weak staining was found in 50% (22 of 44) of CRPC cases (Figure 1C, image 3). The remainder of CRPC cases was negative for AKR1C3 (Figure 1C, image 4). The immunohistochemical staining also showed that AKR1C3 protein was predominantly expressed in cancer (epithelial) cells and not in the stromal cells (Figure 1C).

**AKR1C3 Expression in PCa Cell Lines**

We measured AKR1C3 expression in several prostate (cancer) cell lines. Of all tested cell lines, DuCaP and VCaP cells had the highest AKR1C3 protein expression (Figure 2). These cell lines are propagated under very low androgen levels (27); hence, they can be considered models mimicking CRPC. Both cell lines express a wild-type AR (28). We selected the DuCaP cell line for further experiments.

**DHEA Stimulated DuCaP Proliferation and DHEA Conversion into T and DHT**

DHEA growth was stimulated in DHEA-supplemented medium. Maximal DuCaP proliferation was reached at 100 nmol/L DHEA (Figure 3B). This concentration was used for further experiments. DuCaP proliferation increased exponentially in 100 nmol/L DHEA-supplemented medium (Figure 3C). Proliferating DuCaP cells were capable of converting DHEA into AND and the potent androgens T and DHT (Figure 3D). No androgens were detected in conditioned media from cells grown without DHEA supplementation at any time point (not shown). AKR1C3 protein levels were downregulated during DHEA-stimulated proliferation (Figure 3E).

**Interfering with AKR1C3 Activity Diminishes Cell Proliferation and Induces Apoptosis**

Recently, an enzyme inhibitor for AKR1C3 (INN) was described (26,29). Treatment of DuCaP cells with INN at 10 μmol/L significantly inhibited cell proliferation at d 16. The more pronounced effect was seen at 25 μmol/L INN (Figure 4A). This result was accompanied by a reduced conversion of DHEA into T and DHT, as shown by the significant accumulation of the precursor molecule AND and the decrease in T and DHT levels in the INN-treated samples at 16 d of treatment (Figure 4B). Induction of caspase-3 and -7 activity, an early apoptotic process, was already observed after 2-h INN treatment (Figure 4C). Cell cycle distribution (that is, percentage of cycling cells in the G1, S and G2/M phase) did not change significantly after 48 h of INN treatment (Figure 4D). However, an INN dose-dependent increasing number of cells appeared as a sub-G1 population, with the latter being an indicator for apoptosis (30) (Figure 4D, arrow).

**DISCUSSION**

Recent studies on steroidogenic enzymes in CRPC have shown that enzymes...
catalyzing conversion of adrenal androgens are upregulated in prostate tumors after ADT. In particular, AKR1C3 has gained attention because of its stronger expression both at the mRNA and protein level in CRPC compared with normal prostate, BPH or primary PCa (8,9,11,31). However, only ~30% of CRPC patients in our study displayed high AKR1C3 expression, which is in agreement with a previous report (11,19). The limitation of our study is the heterogeneity of previous hormonal treatment of the CRPC group. So further validation using tissue from CRPC patients treated according to standard protocols is needed.

Our studies have shown that AKR1C3 mRNA and protein can be easily detected by PCR and immunohistochemistry in biopsy and transurethral resection of the prostate material, respectively.

Even though our samples were divided into two groups—frozen (RNA and Western blot analysis) and paraffin embedded (immunohistochemistry)—a correlation between AKR1C3 mRNA and protein level was consistent. Immunohistochemistry showed that AKR1C3 was expressed specifically in the carcinoma cells and not in the stromal cells. The specific upregulation of AKR1C3 in a subset of CRPC cases makes it an attractive biomarker to select patients for therapeutic intervention with AKR1C3.

We selected DuCaP as a model for in vitro studies, because DuCaP cells express AKR1C3 at high levels, and the cells express a wild-type AR (28). Wild-type AR only can be activated by T and/or DHT at nanomolar concentrations. Therefore, DuCaP cells fail to proliferate when they are cultured in medium supplemented with DHEA as the sole androgen source, unless DHEA is first converted into T and DHT. We show that DuCaP cells hardly grow in steroid-free medium and that growth was stimulated by adding DHEA. We also provided evidence that DHEA is converted by the tumor cells into T and DHT. This conversion could be blocked by inhibiting AKR1C3 enzymatic activity with a specific inhibitor INN.

Our data showed a downregulation of the AKR1C3 protein upon increasing T and DHT levels. Furthermore, AKR1C3 expression in the DuCaP-N cell line, a DuCaP derivative cell line continuously cultured in the presence of higher androgen level (1 nmol/L R1881), expresses threefold lower AKR1C3 protein than parental DuCaP cells. Consistent with our studies, it was recently demonstrated by
others that addition of synthetic androgens had a negative effect on the expression of AKR1C3 (8). Therefore, high AKR1C3 expression can be considered as a marker of early adaptation to low androgen levels by using the adrenal androgen DHEA.

Interfering with AKR1C3 activity by using INN resulted in an accumulation of AND, because AKR1C3 no longer catalyzes the reduction of AND to form T. As a result of AKR1C3 inhibition, T and DHT levels decreased and cell proliferation ceased. The decrease in viable cells upon INN treatment was caused by the induction of apoptosis, but was not associated with a change in cell cycle progression. Reduced androgen levels have already been related to apoptosis induction. (32) We were not able to measure androstenediol generated by AKR1C3-mediated reduction of DHEA because of radioimmunoassay sensitivity limitations. To the best of our knowledge, this is a first study showing interference of adrenal androgen DHEA conversion through the AKR1C3 enzyme.

Inhibition of AKR1C3 enzyme activity holds promise for the treatment of CRPC patients in the future. One of the challenges in developing an AKR1C3 inhibitor is its >86% sequence identity with the other human members of the AKR1C subfamily, AKR1C1 and AKR1C2. The latter two enzymes will reduce DHT to less active metabolites, 3α-androstenediol and 3β-androstenediol. Therefore, inhibition of these AKR1C activities would inhibit DHT turnover and hence promote proliferative signaling in the prostate (26). Several compounds have been developed and modified from several analogs of nonsteroidal antiinflammatory drugs (NSAIDs) that effectively inhibit AKR1C3 in biochemical assays (33–36). Before INN or other compounds can be used in CRPC patients, the efficacy, toxicity and AKR1C3 specificity of these compounds need to be determined in cell culture and in vivo experiments.

Abiraterone, a specific CYP17A1 enzyme inhibitor reducing adrenal androgen production, has shown a beneficial effect as secondary treatment in CRPC patients (14,37). This treatment, however, will also accumulate steroids with mineralocorticoid properties upstream of CYP17A1, resulting in its side effects, such as hypertension, hypokalemia and fluid overload. To prevent these side effects, abiraterone needs to be co-administered with glucocorticoids and a mineralocorticoid inhibitor. Recent studies show that prednisolone (a glucocorticoid) and mineralocorticoid inhibitors, such as eplerenone and spironolactone, can activate both wild-type and mutant AR, which could lead to drug resistance (38). Inhibition of AKR1C3, might have less side effects than CYP17A1 inhibition in the clinical setting, because AKR1C3 acts further downstream in the androgen biosynthetic pathway and thus will not lead to glucocorticoid reduction and mineralocorticoid accumulation.

CONCLUSION

We show that AKR1C3 can be a biomarker to identify CRPC patients who may benefit from therapies interfering with AKR1C3 activity. Those patients having high levels of AKR1C3 may be amenable to novel forms of secondary hormonal treatment. CRPC tumor cells can convert adrenal androgens into active growth-stimulating androgens (T and DHT), which can be blocked by
interfering with AKR1C3 protein activity. Therefore, novel AKR1C3 targeting drugs may be potential drugs for second-line hormonal treatment of CRPC patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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