Identification and Characterization of MEL-3, a Novel AR Antagonist That Suppresses Prostate Cancer Cell Growth

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Abstract

Antiandrogens are an important component of prostate cancer therapy as the androgen receptor (AR) is the key regulator of prostate cancer growth and survival. Current AR antagonists, such as bicalutamide and hydroxyflutamide, have a low affinity for the AR and as a result block AR signaling insufficiently. Moreover, many patients develop a resistance for bicalutamide or hydroxyflutamide during therapy or show a clinical improvement after withdrawal of the antiandrogen. New and more effective AR antagonists are needed to ensure follow-up of these patients. We therefore developed a screening system to identify novel AR antagonists from a collection of compounds. MEL-3 [8-(propan-2-yl)-5,6-dihydro-4H-pyrazino[3,2-jk]carbazole] was selected as potent inhibitor of the AR and was further characterized in vitro. On different prostate cancer cell lines MEL-3 displayed an improved therapeutic profile compared with bicalutamide. Not only cell growth was inhibited but also the expression of androgen-regulated genes: PSA and FKBP5. Prostate cancer is often associated with mutated ARs that respond to a broadened spectrum of ligands including the current antiandrogens used in the clinic, hydroxyflutamide and bicalutamide. The activity of two mutant receptors (AR T877A and AR W741C) was shown to be reduced in presence of MEL-3, providing evidence that MEL-3 can potentially be a follow-up treatment for bicalutamide- and hydroxyflutamide-resistant patients. The mechanism of action of MEL-3 on the molecular level was further explored by comparing the structure-activity relationship of different chemical derivatives of MEL-3 with the in silico docking of MEL-3 derivatives in the binding pocket of the AR. Mol Cancer Ther; 11(6); 1257–68. ©2012 AACR.

Introduction

Androgen deprivation and the use of androgen receptor (AR) antagonists are indispensable palliative treatments of metastasized prostate cancer (1). Both therapies aim at blocking the activity of the AR, which is the key regulator of androgen signaling and which is involved in growth and survival of prostate cells (2, 3). Whereas androgen deprivation aims at lowering the levels of androgens present in the prostate, the classical AR antagonists or antiandrogens directly compete with testosterone (T) and dihydrotestosterone (DHT) for binding to the AR.

After an initial successful response of the tumor to androgen blocking therapy, different AR-related resistance mechanisms develop as a response to the selective pressure of the therapy, causing the onset of castration-resistant forms (4, 5). Besides increased AR protein levels due to AR gene duplication or increased transcription also other mechanisms such as an imbalance of coactivators versus corepressors, intracrine androgen biosynthesis, and somatic mutations in the AR allow the tumor to survive low levels of androgens (5, 6). The unraveling of these therapy-induced survival mechanisms of prostate cancer cells resulted in a renewed interest in the AR as a target in the late stages of prostate cancer (7).

Bicalutamide and hydroxyflutamide are widely used as standard antiandrogen therapy in the clinic. Nevertheless, they bind the AR with a rather low affinity and are known to induce escape mechanisms (8). Moreover, in settings of increased AR mRNA and/or altered recruitment of AR coactivators or corepressors, they are acting as partial agonist (6, 9). In some cases, discontinuation of therapy led to an improvement of the patient; this phenomenon was named the antiandrogen withdrawal syndrome. Long-term treatment with antiandrogens can also lead to AR point mutations, with a higher frequency in metastatic lesions compared with primary tumors (10, 11). Two mutant ARs, AR W741C and AR T877A, have originated in patients treated with...
bicalutamide and hydroxyflutamide. Functional tests of the receptor pointed out that these mutant ARs have broadened ligand specificity or are activated instead of inactivated by the antiandrogen (12–14). New and more effective therapies that also affect these mutant ARs are needed.

Recently, a new inhibitor of androgen biosynthesis, named abiraterone was approved for treatment of metastatic castration-resistant prostate cancer patients who previously received chemotherapy (15). A new generation of AR antagonists has evolved from the need for more effective and long-term AR inhibition such as RD162 and its derivatives MDV3100 and ARN-509 (16, 17). They inhibit the AR not only by binding with higher affinity but also by preventing nuclear translocation (16). Besides the ligand-binding pocket, other binding sites for AR antagonists have been reported. EPI-001, for example, is directed toward the aminoterminal domain of the AR, which is important for transactivation (18).

The interest in AR antagonists is reflected in the numerous efforts to identify compounds via screening assays. In vitro screenings have been based on ligand-binding assays, reporter gene assays, or conformational changes induced by the compound (19–26). In vivo screens, directly observing effects on androgen-regulated tissues in mice or rats, and in silico screens, combining biologic data with computational methods, have also been used (27–29). Acknowledging screening assays as a powerful tool for isolating new compounds, we have set up a cellular miniaturized androgen-inducible reporter gene to detect AR antagonists in a collection of compounds. MEL-3, comprising a novel nonsteroidal scaffold, was detected and its antagonistic profile was explored further. MEL-3 is not only able to inhibit the growth of AR-positive prostate cancer cell lines, but it can also inhibit the transactivation capacity of 2 mutant ARs (T877A and W741C), indicating that MEL-3 is viable as a second-generation AR antagonist.

Materials and Methods

Chemical compounds

A small collection of compounds (6,500) was provided by the Centre of Drug Design and Discovery of the KU Leuven. It consists of small molecules (<500 g/mol) with a purity of more than 90%, purchased from commercial suppliers. RD162 was purchased from Helix&Bond Pharmaceuticals. Bicalutamide (Casodex) was provided by AstraZeneca. Compounds were dissolved in dimethyl sulfoxide (DMSO; Acros Organics).

Cell culture

Different cell lines, including LAPC4, LNCaP, VCaP, and PC3, were used as validated models for the different types and stages in the progression of prostate cancer (30). LNCaP, PC3, and COS-7 cells were obtained from the American Type Culture Collection, whereas VCaP and LAPC4 were a kind gift from the Laboratory for Experimental Medicine and Endocrinology (KU Leuven) and were authenticated by short-tandem repeat DNA profiling by Genetica. HEK293 was provided by the Laboratory of Biosignaling and Therapeutics (KU Leuven) in 2004. The HEK293 Flp-In cell line used to generate the screening cell line was kindly provided by Prof. Jieming Wong (Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX) in 2006. These cell lines were not authenticated by our laboratory after receiving them. LNCaP, LAPC4, and PC3 were cultured in RPMI-1640, IMDM, and Dulbecco’s Modified Eagle’s Medium (DMEM)/F-12, respectively (Sigma-Aldrich), whereas VCaP, HEK293, COS-7, SelARE, and CIARE were cultured in DMEM. Media were supplemented with 10% fetal calf serum (FCS; Invitrogen) or 5% charcoal-stripped serum (CSS), l-glutamine (Invitrogen), and Penicillin–Streptomycin (Invitrogen).

Medium-throughput screening

SelARE cells were seeded in 384-well plates (Cultur-Plate384; PerkinElmer) at a density of 20,000 cells per well. Cell seeding and liquid handling was carried out by a robotic platform (Freedom EVO; Tecan). Compounds (0.1 mg) were first dissolved in DMSO (Acros Organics) and then diluted in DMEM with 5% CSS and 10 nmol/L DHT to a concentration of 20 μg/mL. Compound dilutions were transferred to the cells in duplicate (final concentration: 2 μg/mL) on each 384-well plate. Cyprot-erone acetate is included as positive control. After overnight incubation, cells were lysed and luminescence was measured using the SteadyLite Plus Reporter Gene Assay system of PerkinElmer. The Z’ factor was calculated for each plate to monitor the robustness of the screening cell line (Z’ > 0.5; ref. 31).

Whole cell competition assay

CIARE cells were treated with a dilution series (0.1 nmol/L to 10 μmol/L) of compound with 1 nmol/L [3H]-labeled mibolerone. After 90 minutes at 37°C, the cells were washed with ice-cold PBS and lysed in 100 μL Passive Lysis Buffer (Promega). After 1 hour of incubation on ice, 75 μL of cell lysate was transferred to a scintillation vial and 2 mL of LumaSafe Plus (PerkinElmer) was added. The amount of [3H]-labeled mibolerone bound to the AR was detected by a liquid scintillation counter (Wallac 1409; PerkinElmer). The compound concentration (BC50) at which 50% of the [3H]-mibolerone is competed from the ligand-binding pocket is calculated using the equation “One site-Fit logIC50” of the nonlinear regression analyses in GraphPad Prism 5 (GraphPad Software).

[3H]-thymidine incorporation assay

VCaP, LNCaP, LAPC4, and PC3 cells were seeded in 96-well plates (Cellstar; Greiner Bio-One). LNCaP cells were serum-starved in RPMI-1640 with 5% CSS for 3 days before stimulation, whereas VCaP, LAPC4, and PC3 cells were stimulated the day after seeding. Cells were grown in medium supplemented with 5% CSS, 10% FCS, 0.1 nmol/L R1881, and different concentrations of the compound of...
interest for 72 hours. Then, [³H]-Thymidine (specific activity: 2 Ci/mmol; PerkinElmer) was added to each well (1 μCi per well). Six hours later, the cells were harvested as described before (32).

CellTiter-Glo luminescent cell viability assay

Compounds were diluted in DMEM with 10% FCS and applied to the cells the day after seeding. Cell viability was measured after 1, 2, 3, and 4 days as instructed by Promega.

Flow cytometric analysis

After 3 days of serum starvation, LAPC4 cells were left to grow for 48 hours in medium with different compounds. The cells were trypsinized, fixed in 70% ethanol and stained with propidium iodide (Sigma) according to a protocol described before (33). Using a Beckman Instruments Coulter Epics XL flow cytometer (Analis), the fractions of cells in each phase of the cell cycle were quantified and analyzed with the System II software (Analis).

Transient transfection and plasmids

After seeding the HEK293 cells and COS-7 cells, the cells were transfected, stimulated overnight, and harvested in Passive Lysis Buffer (Promega). The luciferase and β-galactosidase activities of the cell lysates were measured and calculated. All protocols and plasmids for assessing activity on mutant ARs and on other steroid receptors were described before (34). The expression plasmids for the VP16 activation domain fused with the hAR-NTD (1-529) and for the DBD-LBD of the hAR (538-919) used in the mammalian interaction assay were described before (35). The VP16 activation domain-hAR (1-919) expression construct was a kind gift of Prof. S. Balk (Department of Medicine, Harvard Medical School, Boston, MA).

Cytoplasmic and nuclear fractionation

After 4 hours of stimulation with compound, CIARE cells were scraped and handled according to the protocol of the Nuclear Extract kit (Active Motif). The presence of the AR, α-tubulin, and histone H3 in both cytoplasmic and nuclear fractions were shown by Western blot analysis.

Western blot analysis

Twenty to 50 μg extract per lane was loaded onto a precast NuPAGE Novex 4% to 12% Bis-Tris Gel (Invitrogen). The proteins were blotted on a polyvinylidene difluoride transfer membrane (GE Healthcare) and proteins of interest were visualized by immunodetection combined with chemiluminescence (Western Lightning Plus-ECL; PerkinElmer). Antibodies against α-tubulin (T5168 from Sigma), AR (in-house antibody against N-terminus of AR), histone H3 (ab8895 from Abcam) were used. Secondary antibodies are conjugated to horseradish peroxidase: Anti-rabbit IgG (P0217 from Dako) and Anti-Mouse IgG (P0260 from Dako).

Quantitative reverse transcriptase PCR

After 18 hours treatment, the cells were collected and washed with PBS. The cell pellet is used to extract total RNA using the RNase Mini Kit from Qiagen. cDNA was generated from RNA (1 μg) with Random Hexamer primers (Fermentas) and RevertAid Reverse Transcriptase (Fermentas). Quantitative Real-Time PCR is carried out using Platinum SYBR Green QPCR Supermix-UDG (Invitrogen). Results are normalized to the housekeeping gene β-actin and each sample was analyzed in triplicate. The sequence of the primers used are as follows: β-actin forward 5'-ACCCCAAGGCCAACCG-3' and reverse 5'-TGACGTGTGCTATCCAGGCTGT-3', PSA forward 5'-GTGAGACCTATGGTGGTGGG-3' and reverse 5'-GAGTCCTTGTATCCACCTCCG-3', FKBP5 forward 5'-AAATCCAAACGGAAGGACAA-3' and reverse 5'-GCCACATCTCTGCAGTCGAA-3' and AR forward 5'-AAGCTGCAAGGTCCTTTC-3' and reverse 5'-TCC-TCCGGAATTTATCAGTTG-3'.

Computational docking analysis

All computations were carried out using MOE (Molecular Operating Environment, The chemical computing group, Montreal). Two receptor structures (in complex with DHT and bicalutamide) were retrieved from the PDB database (entries 1T5Z and 1Z95, respectively) and optimized using the protonate 3D algorithm. Subsequently, MEL-3 was docked into the AR using the Triangle Matcher docking algorithm and the London dG scoring function.

Results

Generation of stable luminescent cell lines for screening

To screen compounds for their AR antagonistic properties, stable cell lines were generated, named SeARE and CIARE, expressing the human AR and responding to androgens by expression of their ARE-regulated luciferase reporter gene. These cell lines were generated in 2 steps: first an expression cassette for the human AR under control of the CMV promoter was integrated in a random location of the genome of the HEK293 Flp-In Host cell line. An AR expressing line was selected and second, the Flp-In system (Invitrogen) was used to integrate a pGLA-luciferase reporter gene under control of an E1B TATA box and 4 copies of the Sp1-HRE (5' AGAACCTTGCAGGACAA-3') or Sp1-HRE 2 mutated (5' AGAACCTTGCAGGACAA-3') in the Flp Recombination Target site. The resulting cell lines were named SeARE and CIARE, respectively.

Validation of the screening cell lines

Expression of the human AR by SeARE and CIARE was confirmed via Western blot analysis (Supplementary Fig. S1). Their sensitivity to known androgens such as T, DHT, and methyltrienolone (R1881) was determined by overnight incubation with a dilution series of the androgen ranging from 1 pmol/L to 0.3 μmol/L. The EC₅₀ of DHT, T, and R1881 for SeARE were 0.36, 1.81, and 0.24 nmol/L,
respectively (Fig. 1A). For ClARE, the response to androgens was almost identical (EC50 of DHT, T, and R1881 were 0.33, 1.43, and 0.23 nmol/L, respectively; Supplementary Fig. S2A). Only when androgen-like substances such as medroxyprogesterone acetate (1 μmol/L), mesterolone (1 μmol/L), 5α-androstenediol (1 μmol/L), and DHT (0.1 μmol/L) were added, the reporter system was activated. Ligands of other nuclear receptors such as aldosterone (1 μmol/L), progesterone (1 μmol/L), estradiol (1 μmol/L), vitamine D (0.1 μmol/L),

Figure 1. Antagonistic activity of MEL-3 is detected by SelARE and ClARE. A, dose–response curve of SelARE to DHT, T, and R1881. The luciferase activity corresponding to maximal response was set at 100%. The EC50 values of DHT, T, and R1881 were 0.36, 1.81, and 0.24 nmol/L, respectively. B, specific response of SelARE to androgen-like substances: 1 μmol/L MPA, 1 μmol/L Mes, 1 μmol/L 5α-Adiol, and 0.1 μmol/L DHT. SelARE did not react to other nuclear receptor ligands: 1 μmol/L Ald, 1 μmol/L Pro, 1 μmol/L E2, 0.1 μmol/L Vit D, 1 μmol/L OH-Cort, and 1 μmol/L Dex. C, activity of MEL-3 and its derivatives on ClARE. Results are given as % of the luciferase activity of ClARE treated with DHT alone. D, structure of MEL-3 and its derivatives. MEL-3.1, MEL-3.2, MEL-3.3, MEL-3.4, MEL-3.5, and MEL-3.6 contain an altered side group at position 8, whereas MEL-3.7 and MEL-3.8 contain a modified pyrazino ring.
hydrocortisone (1 μmol/L), and dexamethasone (1 μmol/L) were unable to stimulate the cell lines (Fig. 1B and Supplementary Fig. S2B).

Medium-throughput screening and hit confirmation
To enable detection of AR antagonists, DHT was coadministered at a submaximal concentration (1 nmol/L) to activate the AR. The hit rate of the screening was 1.3% with a false positive rate of only 0.1%. This led to the identification of 8-(propan-2-yl)-5,6-dihydro-4H-pyrazino[3,2,1-jk]carbazole, further called MEL-3, which inhibited more than 95% of the DHT-induced luciferase activity and was therefore selected for further study (Fig. 1C).

Structure–activity relationship of the MEL-3 compound
To gain more information on the molecular binding mode, chemical derivatives of MEL-3 were pursued and tested (Fig. 1C and 1D). In MEL-3.1 to MEL-3.6, the isopropyl group at position 11 of MEL-3 is altered, whereas the aromatic pyrazine of MEL-3 is modified in MEL-3.7 and MEL-3.8 (Fig. 1D). Using computational docking, the observed structure–activity relationship was analyzed from a structural point of view. MEL-3 was docked in the binding pocket of the AR ligand–binding domain (LBD) based on the binding of DHT to the AR LBD and on the binding of bicalutamide to the AR LBD (W741L; refs. 36, 37). The common binding mode for these potent compounds is depicted in Fig. 2A. The key interaction is formed by the free electron pair of nitrogen-16 in the MEL-3 derivatives with Arg-752 of the AR. This interaction overlaps with the interaction of the cyano group in bicalutamide and the carbonyl of DHT and can be considered essential for the binding of the compounds. Methylation of nitrogen-16 in MEL-3.7 prevents formation of this hydrogen bond as the free electron pair conformation has changed and the space is now occupied with a proton, whereas in MEL-3.8, reduction of the pyrazine into a piperazine disables hydrogen bond formation with Arg-572 due to the incorrect orientation of the free electron pair of nitrogen-16. The disturbance of this hydrogen bond therefore results in the absence of potency of MEL-3.7 and in the inactivation of MEL-3.8. The hydrophobic cyclohexyl moiety of the MEL-3 derivatives is overlapping nicely with the hydrophobic trifluoromethyl group of bicalutamide. Furthermore, the activity of MEL-3.1, containing a methyl group at position 11, on CIARE is comparable with the activity of MEL-3 itself. Other alterations into chloride (MEL-3.2), bromide (MEL-3.3), cyclohexyl (MEL-3.4), isoxyoxy (MEL-3.5), and benzyloxy (MEL-3.6) led to decreased AR antagonistic properties. The preference for hydrophobic groups at position 11 can be explained by the localization of the isopropyl and methyl function of MEL-3 and MEL-3.1 in a hydrophobic area of the ligand-binding pocket. The compounds with different, nonhydrophobic substituents at position 11 can still bind the receptor, but their binding mode is distorted, explaining the drop in potency.

MEL-3 targets the transactivation mechanism of the AR
Several indispensable properties of AR functioning such as DNA binding, N/C interaction, and cellular localization could be affected by MEL-3. To examine whether the MEL-3–AR complex is able to bind DNA once it is in the nucleus, a fusion protein of VP16 and AR was used. This VP16–AR is independent of the ligand-induced conformational changes needed for nuclear translocation and coactivation (38). MEL-3 acts as an antagonist on AR wt and it inhibits the DNA binding of the VP16–AR fusion protein comparable with RD162 (Fig. 2B).

MEL-3 also inhibits the N/C interaction of the AR induced by R1881, as was shown in a mammalian protein interaction assay. Interaction between VP16-NTD and DBD-LBD of the AR was measured by detection of the luciferase activity generated by an androgen-regulated luciferase reporter gene (Fig. 2C).

Agonists such as R1881 induce a conformational change in the AR LBD that leads to nuclear import of the AR where it elicits its function (39). MEL-3, however, as an antagonist, fails to induce this nuclear translocation of the AR as is seen by the reduced amount of AR in the nuclear fractions of CIARE cells that have been stimulated with MEL-3 for 4 hours (Fig. 2D).

Potency of MEL-3 and affinity for the AR
The potency of MEL-3 for inhibiting DHT-responsive transactivation in CIARE was evaluated in 2 ways. In the experiment depicted in Fig. 3A, the concentration of MEL-3 was varied in presence of 1 or 10 nmol/L DHT, which showed the dose-dependent AR inhibitory effect of MEL-3 and MEL-3.1. Importantly, no agonism was observed on CIARE (Supplementary Fig. S3). In Fig. 3B, the DHT concentration was varied in presence of 1 or 10 μmol/L MEL-3. The agonistic signals of up to 10 nmol/L DHT can be reduced to less than 10% by 10 μmol/L MEL-3, whereas 1 μmol/L is not potent enough to do so. In these assays, the antagonistic activity of MEL-3 is comparable with the effects of RD162. MEL-3.1 is a weaker antagonist than MEL-3 and bicalutamide is the weakest of the 4 compounds.

The relative affinities of MEL-3, MEL-3.1, RD162, and bicalutamide for the AR were determined in whole cell competition assays with [3H]-labeled mibolerone (Fig. 3C); their BC50 is 0.83, 1.46, 0.43, and 2.86 μmol/L, respectively. The hierarchy for AR affinity is thus bicalutamide.<MEL-3.1.<MEL-3.<RD162.

MEL-3 activity against hydroxyflutamide- or bicalutamide-resistant AR mutants
The effect of MEL-3 on the transactivation capacity of 2 mutant ARs (AR T877A and AR W741C) was investigated to evaluate the potential effectiveness of MEL-3 as successive therapy in these cases of therapy resistance. Agonistic and lack of antagonistic effects of hydroxyflutamide
and bicalutamide on AR T877A and AR W741C, respectively, were shown in Fig. 4. MEL-3 and MEL-3.1 maintained their antagonistic effects on both mutant receptors (Fig. 4A). MEL-3, however, has partial agonistic effects on AR T877A in a dose-dependent manner, which could explain why it did not fully antagonize the DHT effects on this mutant (Fig. 4B). MEL-3.1 is less an agonist to AR T877A than MEL-3.

**Effect of MEL-3 on androgen-regulated genes**

The mRNA levels of the endogenous androgen-regulated genes PSA and FKBP5 were determined in LAPC4, VCaP, and LNCaP cells after 18 hours of treatment with R1881 and antagonists (Fig. 5). R1881 was able to increase the basal levels of PSA and FKBP5 in LAPC4 by approximately 19-fold and 3-fold, respectively. MEL-3, RD162, and bicalutamide blocked the R1881-induced expression of PSA and FKBP5 (Fig. 5). Similar results were seen in VCaP cells. MEL-3 and RD162 showed a comparable activity in both cell lines. Bicalutamide however seemed less effective in VCaP. In LNCaP cells that contain the T877A mutation in the AR, the effect of MEL-3 on the R1881-induced expression of PSA and FKBP5 was less pronounced and more comparable with the effect of bicalutamide, whereas RD162 could decrease the levels even further.
**MEL-3 affects proliferation of AR-positive prostate cancer cells**

On the basis of cell viability determinations, a dose-dependent decrease of VCaP proliferation is demonstrated for MEL-3 (Fig. 6A). This effect increases in time but is less pronounced when compared with the effect of RD162. MEL-3.1 and bicalutamide, however, do not seem to affect the growth of VCaP.
In a [3H]-thymidine incorporation assays, the inhibitory effect of MEL-3 on VCaP proliferation was confirmed (Fig. 6B). In this assay, MEL-3.1 also lacks proliferation inhibitory effects, as 10 µmol/L MEL-3.1 can only reduce the FCS-induced growth to 92% (data not shown). Besides its effect on VCaP, MEL-3 is also able to reduce the R1881-induced proliferation of LAPC4 to 4% (Fig. 6B). Flow cytometric analysis of the cell-cycle distribution of MEL-3–treated LAPC4 cells indicated a significant accumulation of cells in sub-G1- and G1 phase and a severe decrease of cells in S phase compared with cells treated with DMSO (Supplementary Table S1). For LNCaP cells that express the AR T877A mutant, the inhibitory effect of MEL-3 is less pronounced and can be compared with the effect of bicalutamide. At 10 µmol/L, MEL-3 can reduce the R1881-induced cell proliferation to 62% (Fig. 6B), whereas bicalutamide and RD162 can inhibit proliferation to 67% and 27%, respectively. The effect of MEL-3 on the proliferation of AR-negative PC3 (Fig. 6B) and HEK293 cells (data not shown) is negligible.

Influence of MEL-3 on AR stability

The AR levels in LNCaP, VCaP, and LAPC4 are unaffected by MEL-3 (Supplementary Fig. S4) when compared with vehicle-treated cells. Quantitative reverse transcriptase PCR pointed out that the AR mRNA levels also remain unchanged by MEL-3 in LAPC4 and LNCaP (Supplementary Fig. S5).

Figure 4. Effect of MEL-3 on mutant ARs. Transient transfection of HEK293 cells with an androgen-responsive luciferase reporter and expression vectors for AR wt, AR T877A, and AR W741C were carried out as described in Materials and Methods. Cells were subsequently treated with hydroxyflutamide, bicalutamide, RD162, MEL-3, and MEL-3.1 in presence of 1 nmol/L DHT (A) or in absence of DHT (B). Data are given relative to the luciferase activity of DMSO-treated cells corresponding to the vehicle control for conditions with 10 µmol/L compound that was set at 100%. Bic, bicalutamide; HOFl, hydroxyflutamide.
The goal of this work was to discover new AR antagonists by means of a cell-based luminescent reporter assay. Stable cell lines, SelARE and ClARE, expressing the human AR and containing an ARE-regulated luciferase reporter gene were established. Both cell lines are highly sensitive to androgens and their response is androgen specific. From a collection of compounds, MEL-3 was identified as potent AR antagonist. Compared with bicalutamide, MEL-3 is a more effective inhibitor of AR activity in the screening cell lines. When compared with RD162, it is slightly less effective. The activity of other steroid receptors such as the glucocorticoid receptor remained unaffected, whereas the activity of the mineralocorticoid receptor and the progesterone receptor were only 2-fold downregulated by MEL-3 (data not shown).

Like RD162 and bicalutamide, MEL-3 competes with mibolerone for binding to the AR (40). The affinity of MEL-3 for AR is intermediary to that of RD162 and bicalutamide, explaining the improved antiandrogenic actions of MEL-3 compared with bicalutamide. This hierarchy (bicalutamide > MEL-3 > RD162) was confirmed in the competition assays on ClARE measuring their antagonistic abilities in presence of different concentrations of DHT.

The fact that several intrinsic functions of the AR such as N/C interaction, DNA binding, and nuclear localization are affected by MEL-3 can explain its antagonistic effects and lack of agonistic effects on AR wt.

**Discussion**

The goal of this work was to discover new AR antagonists by means of a cell-based luminescent reporter assay. Stable cell lines, SelARE and ClARE, expressing the human AR and containing an ARE-regulated luciferase reporter gene were established. Both cell lines are highly sensitive to androgens and their response is androgen specific. From a collection of compounds, MEL-3 was identified as potent AR antagonist. Compared with bicalutamide, MEL-3 is a more effective inhibitor of AR activity in the screening cell lines. When compared with RD162, it is slightly less effective. The activity of other steroid receptors such as the glucocorticoid receptor remained unaffected, whereas the activity of the mineralocorticoid receptor and the progesterone receptor were only 2-fold downregulated by MEL-3 (data not shown).

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The fact that several intrinsic functions of the AR such as N/C interaction, DNA binding, and nuclear localization are affected by MEL-3 can explain its antagonistic effects and lack of agonistic effects on AR wt.
To explain antagonistic activity of MEL-3 at the molecular level, a limited structure–activity relationship was set up. Prompted by the observed agonistic effects of MEL-3 on AR T877A, which is mutated in the ligand-binding pocket, an in silico model of its binding mode in the pocket of the AR was calculated. These in silico data explain why alterations of nitrogen-16 in the pyrazine ring of MEL-3 are deleterious to its antagonistic activity and why on the other hand differences in the side chain at position 11 only diminish the potency and the antagonistic activity as the side groups are becoming larger or less hydrophobic. The hydrogen bond formed between nitrogen-16 of MEL-3 and Arg-752 of the AR is a key interaction for AR binding because similar hydrogen bonds involving Arg-752 or...
Glu-711 are seen for every AR ligand. It should be noted, however, that it cannot be formally excluded that MEL-3 binds to sites elsewhere in the AR-LBD (41).

Not only the androgen-regulated expression of luciferase in SelARE is affected by MEL-3 but also the expression of endogenous androgen-regulated genes such as PSA and FKBP5 in AR-positive cell lines are decreased. Importantly, this reduced gene expression cannot be explained by decreased levels of AR protein. The specific decrease in expression of androgen-regulated genes and the unaffected expression of a constitutive luciferase gene (data not shown) clearly point out that the AR is the target of MEL-3.

Ineffectiveness of AR antagonists can arise by the occurrence of mutations in the ligand-binding pocket of the AR, which will eventually lead to therapy resistance. MEL-3 effectively inhibits 2 mutant ARs (T877A and W741C) that have been detected in prostate cancer patients treated with bicalutamide and hydroxyflutamide. The lack of a dose-dependent antagonistic effect on the transcriptional activity of AR T877A and the weak inhibitory effects on proliferation of LNCaP could be explained by the partial agonistic effects of MEL-3 on AR T877A.

To further describe the therapeutic potential of MEL-3, we tested its inhibitory effect on the proliferation of VCaP, LAPC4, and LNCaP. For LAPC4, MEL-3 showed stronger inhibitory effects compared with bicalutamide and RD162; whereas for VCaP and LNCaP, MEL-3 inhibited proliferation better than bicalutamide, but not as good as RD162. Notably, in a MTT assay MEL-3 showed no cytotoxic effects on any of these cell lines at concentrations and time points used in the experiments described above (data not shown). Accumulation of LAPC4 cells in G1 is a first indication that this growth inhibitory effect is regulated by the AR as it is a known regulator of the G1- to S phase progression in prostate cells (2). In addition, the proliferation of AR-negative PC3 cells remained unaffected by MEL-3.

Despite its pronounced effect on cell proliferation in 2 independent assays, MEL-3 does not seem to induce apoptosis via the caspase-3 cleavage pathway (Supplementary Fig. S4). Caspase-independent mechanisms of apoptosis, however, remain to be investigated.

In summary, our data show that MEL-3 represents a new structural class of AR antagonists. It not only inhibits AR signaling leading to decreased expression of androgen-regulated genes but it also inhibits the proliferation of androgen-dependent and androgen-independent AR-positive prostate cancer cell lines. MEL-3 binds the AR with higher affinity compared with bicalutamide, which can explain its improved performance in cell proliferation assays. Patients who developed therapy resistance for bicalutamide due to the AR W741C mutation might benefit from treatment with MEL-3. In addition, our in silico data predict that MEL-3 is able to bind the ligand-binding pocket of the AR via a hydrogen bond with Arg-752. Decreased activity or lack of activity of several chemical derivatives of MEL-3 can be structurally explained by interfering with the most optimal binding mode predicted for MEL-3. Whether MEL-3 induces an alternative conformation of the AR-LBD and thereby initiates a unique mechanism of inhibition reflected by its increased efficacy compared with bicalutamide remains to be determined. Structural analyses of MEL-3 in complex with the AR-LBD will therefore provide new information that will certainly contribute to the development of better AR antagonists.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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