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A novel steroidal antiandrogen targeting wild type and mutant androgen receptors

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ABSTRACT

Prostate cancer (PCa) progression is enhanced by androgen and treatment with antiandrogens represents an alternative to castration. While patients initially respond favorably to androgen ablation therapy, most experience a relapse of the disease within 1–2 years by expressing androgen receptor (AR) mutants. Such mutations, indeed, promote unfavorable agonistic behavior from classical antagonists. Here, we have synthesized and screened 37 novel compounds derived from dihydrotestosterone (DHT), cyanolutamide and hydroxyflutamide. These derivatives were tested for their potential antagonistic activity using a luciferase reporter gene assay and binding properties were determined for wild type (WT) and mutant ARs (T877A, W741C, W741L, H874Y). In the absence and presence of antiandrogens, androgen dependent cellular proliferation and prostate specific antigen (PSA) expression were assayed in the prostate cancer cell line LNCaP by crystal violet, real time PCR and by Western blots. Also, cellular proliferation and PSA expression were assayed in 22Rv1. A novel compound RB346, derived from DHT, was found to be an antagonist for all tested AR forms, preventing DHT induced proliferation and PSA expression in LNCaP and 22Rv1 cells. RB346 displayed no agonistic activity, in contrast to the non-steroidal antiandrogen bicalutamide (Casodex®) with unfavorable agonistic activity for W741L-AR. Additionally, RB346 has a slightly higher binding affinity for WT-AR, T877A-AR and H874Y-AR than bicalutamide. Thus, RB346 is the first potent steroidal antiandrogen with efficacy for WT and various AR mutants.

1. Introduction

It has been known for more than 70 years that prostate cancer progression is stimulated by agonistic androgen action [1]. The first line treatment for clinically localized disease is radical prostatectomy. In an advanced stage, the initial treatment for metastatic PCa consists in androgen-deprivation therapy (ADT) by medical castration using luteinizing hormone-releasing hormone (LHRH) agonists or by surgical castration with bilateral orchiectomy [2]. By blocking the androgen of testicular but also of adrenal origin, androgen receptor (AR) antagonists represent an efficient alternative as monotherapy or combined to castration [2]. Defined by the chemical structure, antiandrogens fall into two classes steroidal and non-steroidal antagonists. The main steroidal antagonists, cyproterone acetate (CPA) (Fig. 1A), chlormadinone and allylestrenol prescribed for the treatment of PCa and prostate hyperplasia are progesterone derivatives. As a consequence, they harbor progestin properties (rev in [3]). Interestingly spironolactone, an aldosterone antagonist, exhibits weak anti-androgenic actions [4]. In contrast, the non-steroidal antagonists, bicalutamide (Fig. 1A), flutamide and nilutamide, are highly selective for AR.

Many patients on antiandrogens became therapy-resistant and suffer from "antiandrogens withdrawal syndrome". Several mechanisms for resistance have been described including ligand-independent activation of AR [5,6], androgen antagonist expulsion through ATP-binding cassette pumps [7], or increased level of AR [8]. Over-expression of the AR modulates the co-repressors to co-activators ratio and as a result some antiandrogens, such as bicalutamide, were unfavorably converted from an AR antagonist to an AR agonist [8].

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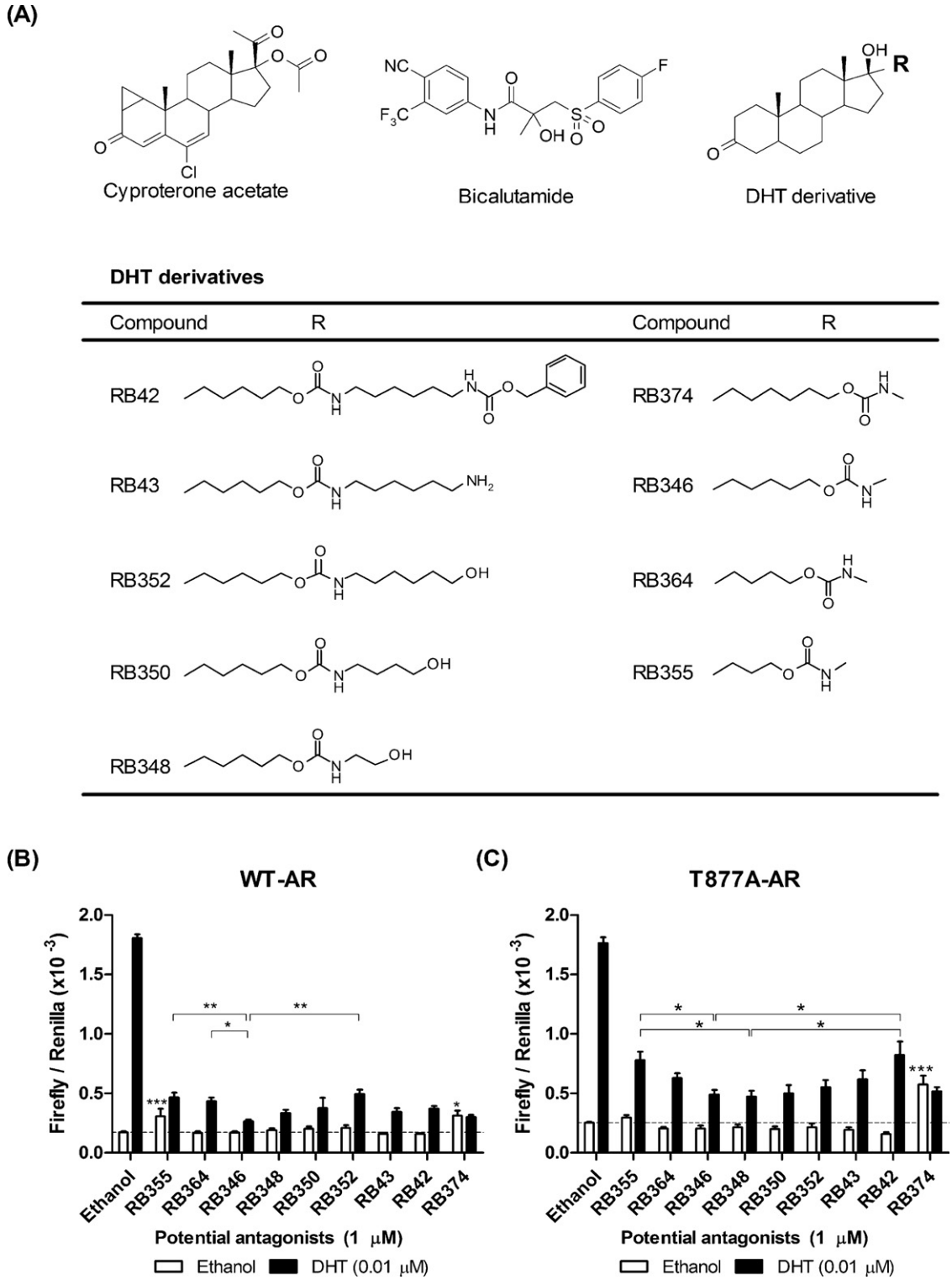

Fig. 1. Structures of AR ligands and their function. (A) Chemical structures of CPA, bicalutamide and of potential DHT derived AR antagonists. (B and C) Agonistic and antagonistic activities of DHT-derivatives in reporter gene assays with WT- and T877A-AR transfected COS7 cells. Cells were treated with vehicle (ethanol) or chemicals only (1 μM) (white bars) for the agonistic mode or with DHT (10 nM) and vehicle or chemicals (1 μM) combined for the antagonistic mode (black bars). Results are mean values (±SEM) of at least three independent experiments performed in triplicate ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Evidence suggests that AR mutations, appearing during antiandrogen treatment, partly account for PCa therapeutic failure [9–11]. The most commonly reported AR point mutations are located in the ligand binding domain (LBD), including substitutions of alanine to threonine at residue 877 (T877A-AR) [12] or histidine to leucine at position 701 (L701H-AR) [13,14] or histidine to tyrosine at position 874 (H874Y-AR). The T877A mutation is present in the LNCaP cell line originally derived from a metastatic lesion of the lymph nodes from a PCa patient [15,16]. More recently mutations at codon W741 (W741C and W741L) were found in LNCaP cells that were kept in long term culture in presence of bicalutamide [9]. Identical mutations were identified in metastatic lesions of patients being treated with bicalutamide [10]. The H874Y mutation was described in CWR22 and in 22Rv1 cells

[17,18]. The 22Rv1 cell line is an androgen responsive and independent human prostate carcinoma cell line derived from a human prostatic carcinoma xenograft CWR22 [19]. This cell line expresses a long form of AR with a duplication of exon 3 affecting the DNA binding domain and a mutation in the ligand binding domain AR (H874Y) [17,20]. Additionally 22Rv1 cells express a short AR isoform resulting from proteolytic cleavage of full-length AR^{Ex3dup} [20]. It was proposed that the truncated form accounts for androgen independent growth, whereas the mutated form accounts for androgen responsiveness [20].

For several decades, the impact of the AR mutations on the efficacy of antiandrogen treatment has been an important domain of research. By altering the specificity of the ligand-binding pocket or the co-activator recruitment [21] the AR mutations may result in the convertion of AR antagonists into agonists, promoting activation where inhibition is desired. One notable example is the activation of the AR mutants T877A, H874Y and T877S by cyproterone [21–23]. Similarly, bicalutamide serves as agonist for the AR mutants T877A, W741C, W741L or H874Y in in vitro experiments [9,24]. In contrast L701H-AR and L701H/T877A-AR were neither activated by CPA nor by bicalutamide [24–26]. Importantly, a comparative study performed in 2007 demonstrated that none of the tested steroidal or non-steroidal antagonists were capable of efficiently antagonizing all AR forms, i.e. WT-AR, W741C, T877A and W741C/T877A-AR [27]. As a consequence switching one antiandrogen to another in the medication-based treatment might achieve an efficient androgen deprivation in case of refractory PCa [27]. The recent promising results obtained with MDV3100 [28] have underlined the interest to synthesize and characterize new molecules exhibiting antagonist properties against the various AR forms.

To the best of our knowledge no efficient antiandrogen had been developed based on the structure of dihydrotestosterone (DHT), the endogenous cognate ligand for the AR. DHT harbors an interesting selectivity for AR. Indeed, when developed for the positron emission tomography imaging, DHT derivatives showed low affinity for other steroid hormone receptors [29]. Additionally, DHT only barely activates the progesterone receptor (PR) whereas progesterone and progesterone derivatives are strong AR agonists [30]. This suggests that DHT represents a potentially effective core structure for the design of new steroidal antiandrogens.

In the present paper we report the screening of a series of newly synthesized DHT derivatives characterized by a bulky side chain attached at the steroidal D ring. The results include the identification of a novel potentially pure antagonist for WT and the clinically relevant T877A, W741C, W741L and H874Y mutated ARs.

2. Materials and methods

2.1. Cell culture

COS-7 cells were maintained in DMEM supplemented with 4.5 g/l glucose (Invitrogen, Basel, Switzerland) 10% fetal bovine serum (FBS) and antibiotics. Similarly human LNCaP and 22Rv1 prostate cancer cells were cultured in RPM-1640 (Invitrogen, Basel, Switzerland), 4.5 g/l glucose, 10% FBS and antibiotics. LNCaP were cultured on poly-D-lysine (Sigma, Buchs, Switzerland) coated wells.

2.2. Plasmids and transfections

The following plasmids were used: pSG5AR containing the full-length cDNA of WT human AR (kind gift of Dr. Palvimo, University of Kuopio, Finland [31]); pMMTV, pPR-alpha, pPR-beta provided by Dr. Rusconi, University of Fribourg, Switzerland and Dr. Chambon Institute for Genetics, Strasbourg, France. pCMV-Renilla was from Promega (Wallisellen, Switzerland). Transfections were performed with FuGENE HD transfection reagent (Roche, Rotkreuz, Switzerland) using 3 μl of solution for 1 μg of plasmid.

2.3. Site-directed mutagenesis

For translocation and binding studies mutated receptors were generated from the pSG5-WT-AR plasmid. We performed site-directed mutagenesis (W741C, W741L, L701H, T877A, L701H/T877A and H874Y) using QuikChange II XL site-directed mutagenesis kit (Stratagene, Basel, Switzerland). The GFP-chimerical mutants for translocation studies were generated similarly. The following primers were used:

5′GACTCCTTTGCAGCCTTGCACTCTAGCCTCAATGAACTGG3′ and 5′CCAGTTCATTGAGGCTAGAGTGCAAGGCTGCAAAGGAGTC3′ for L701H, 5′GCTGCATCAGTTCGCTTTTGACCTGC3′ and 5′GCAGGTCAA GCAGGTCAA AAGCGAACTGATGCAGC3′ for T877A, 5′CTGTCAT TCAGTACTCCTGTATGGGGCTCATGGTGTTTG3′ and 5′CAAACACCAT GAGCCCCATACAGGAGTACTGAATGACAG3′ for W741C, 5′CTGTCATT CAGTACTCCTTGATGGGGCTCATGGTGTTTG3′ and 5′CAAACACCATG AGCCCCATCAAGGAGTACTGAATGACAG3′ for W741L, 5′CTATTGCG AGAGAGCTGTATCAGTTCACTTTTGACCTGC3′ and 5′GCAGGTCAAAA GTGAACTGATACAGCTCTCTCGCAATAG3′ for H874Y.

Underlined and bolded types represent nucleotide exchange introduced into the WT sequence. All plasmids were controlled by DNA sequencing (Microsynth, Balgach, Switzerland).

2.4. Receptor competitive binding assay

COS-7 cells cultured in 24 well plates (density 50,000 cells/well) were transfected with 500 ng of receptor encoding plasmids 24 h after plating. One day later, washed cells were cultured in 0.5 ml of phenol-red free DMEM (Invitrogen, Basel, Switzerland) with 2% charcoal treated serum (CT-FBS, Perbio, Thermo Fisher Scientific, Lausanne, Switzerland) for additional 24 h. Transfected and washed cells were maintained in 0.5 ml DMEM-2% CT-FBS for 2 h [17α-methyl-^{3}H]-mibolerone (83.3 Ci/mmol, 20 pM) (Perkin Elmer, Schwerzenbach, Switzerland) or [*1,2,6,7*-^{3}H]-progesterone (94 Ci/mmol, 40 pM) (Amersham, Otelfingen, Switzerland) and various compounds in ethanol (0.1% final concentration) were added to cells for 1 h. Supernatants were subsequently collected and receptor bound steroids extracted with 0.3 ml of 80% ethanol for 1 h. Both fractions were mixed with scintillation liquid (Irgasafe, Perkin Elmer, Schwerzenbach, Switzerland) and radioactivity counted (Tri-Carb 2000CA, Canberra Packard, Vienna, Austria). The percentage of binding in presence or absence of derivative was calculated relatively to total dpm and results expressed as percentage of binding relative to the ^{3}H-tracer alone (100%).

2.5. Translocation assay

COS-7 cells seeded on cover slides in 12 well plates (density 60,000 cells/well) were transfected using 0.5 μg of GFP-chimerical AR plasmid per well. Cells in phenol-red free DMEM with 2% CT-FBS were treated with DHT or derivatives 24 h later for 1 h. Washed cells were fixed (PFA 2%, 30 min). Cover slides were mounted with Mowiol (Merck, Darmstadt, Germany) and analyzed by fluorescent microscopy.

2.6. Transactivation assay

For reporter gene assays, COS-7 plated in 48 well plates (density 10,000 cells/well) were transfected with 300 ng of pMMTV-Firefly, 5 ng of pCMV-Renilla and 25 ng of receptor encoding plasmid. Six hours later washed cells were cultured in phenol-red free DMEM

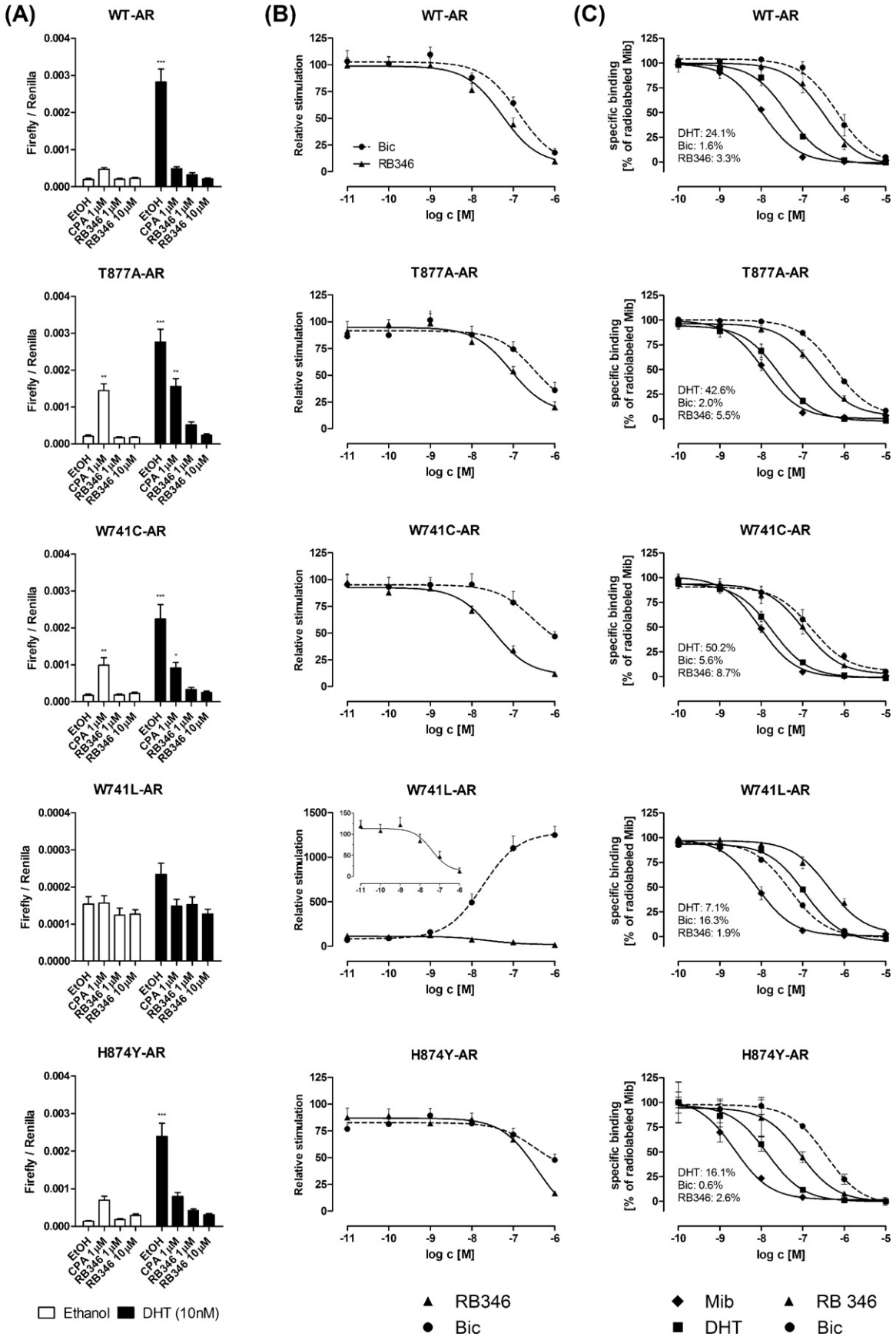

Fig. 2. Binding characteristics and impact of RB346 on transactivation potential of WT-AR and mutant AR forms. (A) Agonistic and antagonistic activities of RB346. COS-7 cells cotransfected with human WT-AR or other mutant ARs, with pMMTV-luc and internal control were treated with DHT (10 nM) or chemicals only (RB346 1 μM or 10 μM and CPA, 1 μM) for the agonistic mode or with DHT (10 nM) and chemicals (1–10 μM) combined for the antagonistic mode. Luciferase activity was assayed 24 h after treatment. Results are averages of at least three independent experiments performed in triplicate. (B) Comparison of AR antagonistic activities of RB346 (▲) and bicalutamide (●) for

with 2% CT-FBS and treated with DHT or derivative compounds 24 h later for additional 24 h. The luminescence of lysed cells was measured in a Fluoroskan luminometer (Thermo Electron, Waltham, MA, USA) using Dual-Luciferase Reporter Assay (Promega, Wallisellen, Switzerland). Luciferase activity was normalized to Renilla activity.

2.7. Proliferation assay

On day 4 RPMI 2% CT-FBS was added with DHT or compound of interest to LNCaP cells cultured in 96 well plates. Ninety-six hours later, cells were fixed (2% PFA). Proliferation was assayed with crystal violet staining. 22Rv1 were cultured in RPMI 10% CT-FBS for 2 days and thereafter treated for an additional 72 h. Proliferation was assayed with Cell Titer Glo assay (Promega, Wallisellen, Switzerland).

2.8. Real-time reverse transcriptase polymerase chain reaction

LNCaP cells seeded in poly-D-lysine coated 6-well plates (300,000/well) received RPMI1640 2% CT-FBS forty-eight hours later. 24 h thereafter cells were treated with DHT or compounds. Total RNA was extracted using SV total RNA isolation system (Promega, Wallisellen, Switzerland), and cDNA synthesized from 1 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, Basel, Switzerland). Primers for human PSA (Hs00426859_g1), PS2 (Hs00907239_m1) and GAPDH (Hs99999905_m1) were used with FastStart DNA Master Mix (Applied Biosystems, Rotkreuz, Switzerland). The ratio of PSA mRNA or PS2 mRNA to GAPDH mRNA was calculated. Experiments were performed in triplicates and repeated at least twice.

2.9. Measurement of prostate-specific antigen (PSA)

Conditioned medium of LNCaP and 22Rv1 cells treated for 24 h and 72 h respectively as described, was collected, centrifuged and stored at −80 °C. Relative cell proliferation was assayed using crystal violet. PSA levels were determined (electrochemiluminescence immunoassay) with a Roche Modular automated platform and were normalized to cells numbers. For Western blots LNCaP cells were lysed in ice-cold RIPA buffer, centrifuged (5 min, 4 °C, 15,000 rpm), boiled and subjected to SDS–PAGE (12%). Blocked membranes were exposed to anti-PSA (sc-7316, SantaCruz, Heidelberg, Germany) and anti-mouse HRP antibodies (sc-2005, SantaCruz, Heidelberg, Germany).

2.10. Induced fit docking

Schrödinger 2010 package induced fit docking protocol (IFD) was used to predict the binding mode of compound RB346 relative to the WT- and the various mutated ARs [32]. IFD is an iterative procedure that combines Glide rigid receptor docking with Prime protein structure prediction in three steps [33,34]. The ligand was docked using Glide SP into the receptor and the ligand-receptor complexes were refined. Prime was used to predict the side-chains proximal to the ligand for optimal orientation, with respect to an induced fit mechanism. The final step included re-docking the ligand into the generated relaxed complexes. The best-ranked model was used for comparison with available crystal structures in the Protein Data Bank (PDB).

The antagonistic behavior of RB346, particularly against two mutants promoting agonism in R-bicalutamide, prompted investigation of binding to three co-crystallized AR LBD structures, WT·DHR (PDB code 1i37), T877A·CPA (PDB code 2oz7) and W741L·R-bicalutamide (PDB code 1z95) [35,36]. All structures were prepared for docking using the standard protocols, by adding bond orders and hydrogen, and removing all waters greater than 5 Å away from the ligand. All crystal structures were aligned to WT-AR structure 1i37 for comparison of binding modes. Compounds were docked into the receptor with all non-polar atoms van deer Waals radius scaled by a factor of 0.5, and 20 reasonable poses are retained.

2.11. Statistics

Data are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed with one-way ANOVA with Bonferroni's post hoc test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Screening of potential antagonists for AR by transactivation assay

In preliminary experiments, we screened 28 DHT-, 7 cyanonilutamide- and 2 hydroxyflutamide- derivatives designed and synthesized in our laboratory using COS-7 cells cotransfected with human WT-AR or T877A-AR plasmids and a firefly luciferase reporter gene plasmid with a murine mammary tumor virus (MMTV) promoter. The DHT derivative RB42, (Fig. 1A) was the most efficient antagonist for both WT-AR and T877A-AR without agonistic property and was therefore considered as lead compound for pure antagonists development.

Based on the structure of RB42, several derivatives with modified side chains were synthesized and assayed to characterize the structural requirements for the antagonist function (Fig. 1A). All compounds tested harbored a relevant antagonist activity for both receptors, with RB346 having the strongest antagonist activity and no measurable agonist effect (Fig. 1B, C). Interestingly, reducing the length of the alkyl side chain from 5 (RB346) to 4 or 3 carbon atoms (RB364 and RB355, respectively) decreased the antagonist activity and the binding affinity for ARs (Fig. 1B, C and data not shown). In contrast, after elongating the length of the alkyl side chain from 5 (RB346) to 6 carbon atoms (RB374) or replacing the carbamate group by a hydroxyl group, the compounds became agonistic for ARs (Fig. 1B, C and data not shown). Together these data suggested that (i) the carbamate group in C5′ position of the side chain was necessary for the pure antagonist function and (ii) the extension and truncation of the side chain reduced the antagonist properties.

3.2. Activity of RB346 on WT and mutated ARs

The antiandrogen activity of RB346 was further studied on W741C-, W741L- and H874Y-AR forms (Fig. 2A). RB346 was an antagonist for all tested AR mutants. Only a small agonistic effect, not reaching significance, was observed for H874Y-AR at high concentration (2.12 fold of stimulation with 10 μM of RB346) (Fig. 2A). In comparison, we confirmed that CPA acted as an agonist for WT-, T877A-, W741C- and H874Y-ARs at a concentration of 1 μM (Fig. 2A) as previously reported [23,27].

WT-AR and mutant AR forms. COS-7 cells cotransfected with various AR forms, pMMTV-luc and internal control were treated with DHT (10 nM) and an increasing concentrations of antagonist. Results are averages of at least three independent experiments performed in triplicate. (C) Competitive whole cell binding assays. Cells transfected with WT-AR, T877A-AR, W741C-AR, T741L-AR or H874Y-AR were incubated with [^{3}H]-mibolerone (20 pM) in the absence or presence of increasing concentration of unlabeled mibolerone, DHT, bicalutamide or RB346 for 1 h. The percent of [^{3}H]-mibolerone specifically bound to the AR is given. Results are averages of at least three independent experiments. The RBA values are given for each receptor with the RBA of mibolerone arbitrary as 100%.

The efficacy of RB346 was compared with the activity of the non-steroidal antagonist bicalutamide (Fig. 2B). RB346 and bicalutamide reduced the DHT-induced reporter activity in a dose dependent manner when WT-AR was over expressed in COS cells (Fig. 2B). Both ligands exhibited a similar activity with a slightly higher potency for RB346. The IC_{50} values were 0.14 μM and 0.054 μM for bicalutamide and RB346 respectively. At a concentration of 1 μM both ligands completely abrogated the DHT-induced reporter activity (Fig. 2B). The results observed with bicalutamide are in agreement with reported IC_{50} values of 0.3–0.9 μM [37]. RB346 and bicalutamide were less efficient in antagonizing DHT-induced reporter activity when T877A-AR mutant was over expressed. At a concentration of 1 μM, RB346 reduced at least 60% of the DHT-induced reporter activity, an effect more pronounced than that of bicalutamide (Fig. 2B). Interestingly, RB346 showed a strong AR antagonistic activity when AR harbors a mutation on the codon 741 (W741C and W741L). In contrast bicalutamide was either a weak W741C-AR antagonist (Fig. 2B) or even a strong W741L-AR agonist (Fig. 2B) an observation in agreement with published data [9,27]. Interestingly RB346 showed a better antagonistic efficacy for H874H-AR than bicalutamide (Fig. 2B).

The activity of RB346 was additionally tested on L701H-AR and the double mutant T877A/L701H-AR (Supplementary Fig. S1). The AR harboring the mutation L701H was almost not responsive to DHT as described [24,38] (Fig. S1). RB346 was a potent antagonist for the mutant T877A/L701H-AR. Interestingly, RB346 did not activate the double mutant T877A/L701H-AR (Fig. S1), whereas this double-mutant is responsive to non-androgens such as progesterone and estradiol, which is explained by its relaxed ligand binding domain [19].

3.3. Competitive whole cell binding assay

The ability of the ligand RB346 to bind to WT-AR and mutant ARs was assayed by displacement of [^{3}H]-mibolerone in COS-7 cells (Fig. 2C). RB346 inhibited the binding of [^{3}H]-mibolerone to WT-AR and mutant ARs, dose-dependently. In comparison with bicalutamide, RB346 showed a slightly higher relative binding affinity (RBA) in COS-7 cells expressing human WT-AR, T877A-AR, W741C-AR and H874Y-AR (Fig. 2C and Supplementary Table S1), while RB346 had a lower RBA for W741L than bicalutamide (Fig. 2C and Table S1). In addition, whole cell binding studies using [^{3}H]-progesterone in the presence of an excess of unlabeled progesterone, CPA or RB346 were performed in PR-expressing COS-7 cells. The IC_{50s} values of progesterone CPA and RB346 were 0.012 μM, 0.029 μM and 0.18 μM, respectively indicating a significantly lower affinity of RB346 for PRβ compared to CPA (Supplementary Fig. S3).

3.4. Impact of RB346 on the modulation of other steroid receptors

The effect of RB346 on the glucocorticoid receptor (GRα), mineralocorticoid receptor (MR) and progesterone receptor (PRβ) activity was also studied (Fig. 3A). COS-7 cells were transfected with the corresponding plasmids encoding the cognate receptor together with the MMTV-luciferase and the CMV-hRL plasmids. The cells were subsequently incubated with the glucocorticoid dexamethasone (0.1 nM), the mineralocorticoid aldosterone (0.1 nM) or progesterone (10 nM) in the absence or presence of RB346 or CPA (1 μM) for 24 h. In this model the steroids increased the luciferase activity 137, 30 and 9 times after dexamethasone, aldosterone or progesterone treatment respectively, whereas RB346 revealed no agonistic function. It is noteworthy that RB346 is a potential antagonist for MR and PRβ demonstrating an inhibition of the steroid induced activity by 75% and 30%

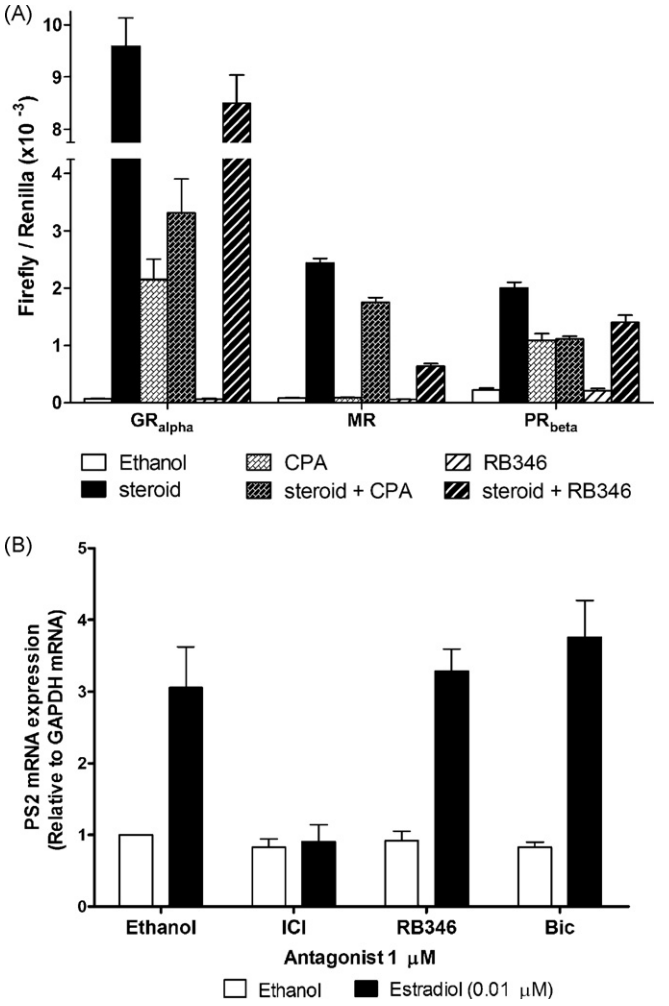

Fig. 3. Assessment of selectivity of RB346 towards other steroid receptors. (A) COS-7 cells cotransfected with human PR_{beta}, GR_{alpha} or MR encoding plasmids combined with pMMTV-luc and internal control were treated with progesterone (10 nM), dexamethasone (0.1 nM), aldosterone (0.1 nM) or RB346 or CPA (each 1 μM) for the agonist mode or with the steroids and chemicals together for the antagonist mode. Results are averages of at least three independent experiments performed in triplicate. (B) Impact of RB346 on mRNA levels of PS2 in MCF-7.

respectively (Fig. 3A), while no antagonist function for GRα was observed (Fig. 3A). CPA activated GRα and PRβ, by 30 and 5 fold, respectively, and showed antagonistic activity for GRα, MR and PRβ with 65%, 30% and 45% inhibition of steroid induced activity, respectively (Fig. 3A).

The impact of RB346 on estrogen receptors (ERα, ERβ) was assayed in MCF-7 cells, expressing both receptors endogenously. The expression of the trefoil factor 1 gene, an estrogen dependent cytokine known as PS2 [39] was monitored by RT-qPCR. The ER antagonist fulvestrant (ICI 182 780) used as reference compound [40] abolished the estradiol induced PS2 mRNA level, while RB346 did not modulate its expression significantly either alone or in combination with estradiol (Fig. 3B).

3.5. RB346 inhibited proliferation of androgen responsive prostate cancer cell lines

In agreement with the literature [41], LNCaP cells responded to DHT in a biphasic dose dependent manner (Supplementary Fig. S2A). A maximum of proliferation (284%) was obtained at 10 pM DHT, while high concentrations stimulated growth only moderately

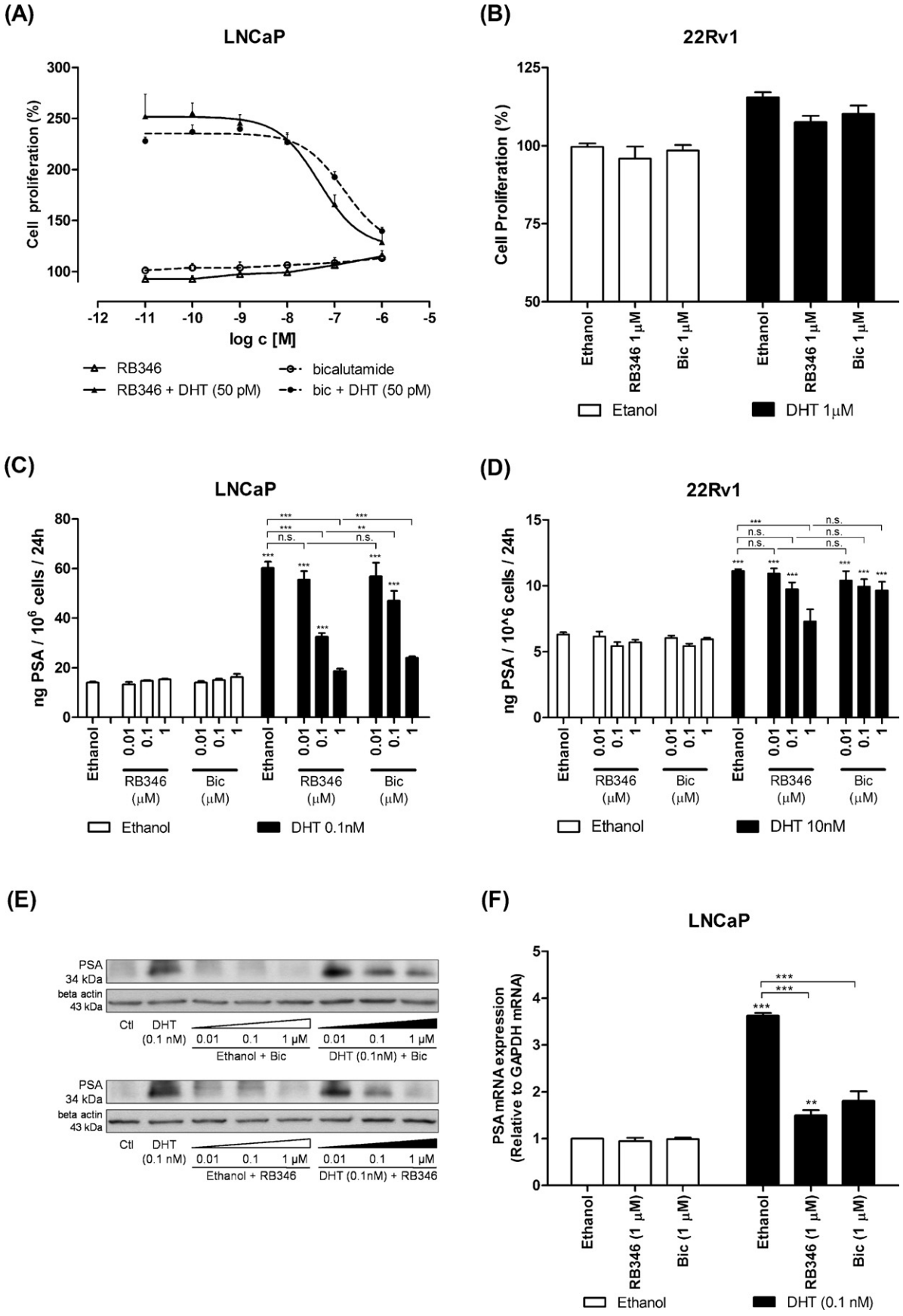

Fig. 4. Impact of RB346 on prostate cell proliferation and on PSA expression. (A, B) Proliferation of LNCAP and 22Rv1 96 h or 72 h after treatment respectively. (C, D) Concentration of secreted PSA in the medium of cultured LNCaP and 22Rv1 cells 24 h or 72 h after treatment respectively (**$p < 0.01$, ***$p < 0.001$). (E) PSA levels monitored by Western blots of cell lysates from LNCaP cells 24 h after treatment. (F) Determination of PSA mRNA expression in LNCaP 24 h after treatment (**$p < 0.01$, ***$p < 0.001$).

(Fig. S2A and Fig. 4A). 22Rv1 cells were less sensitive to DHT but responded to high concentration (110% at 1 μM) (Fig. 4B).

In order to investigate the antagonistic function of RB346 in prostate cancer cell lines prostatic cells in the presence of the endogenous ARs, the proliferation of LNCaP and 22Rv1 cell lines was monitored with an optimal constant DHT concentration and increasing concentrations of RB346 (Fig. 4A and B respectively). Bicalutamide was used for comparison (Fig. 4A). In both cell lines RB346 clearly counterbalanced the effect of DHT while 1 μM RB346 almost completely abrogated the DHT dependent LNCaP growth (Fig. 4A and B respectively). By these measures, RB346 displayed efficacy equivalent to bicalutamide. Notably, the down regulation of LNCaP and 22Rv1 cell proliferation by RB346 is observed only in DHT induced conditions (Fig. 4A and B respectively). 22Rv1 cell line does not express PR and ER suggesting that the androgen response is mediated through the AR [42]. Similarly, RB346 did not modulate the proliferation of DU145, an AR negative prostate cancer cell line and of PC-3, an androgen independent prostate cancer cell line (data not shown). Those data show a specific effect of the compound RB346 on AR activated proliferation pathway. The steroidal antagonist CPA, an agonist of T877A-AR, strongly enhanced the LNCaP cell proliferation (369% at 1 μM) (Supplementary Fig. S2B), a known effect [41].

3.6. RB346 decreased prostate specific antigen (PSA) production in prostate cancer cell lines

The promoter of PSA contains several androgen response elements and is therefore inducible by DHT. The level of secreted PSA was monitored in the media of cultured LNCaP and 22Rv1 cell lines (Fig. 4C and D respectively). The PSA levels in the culture medium from 22Rv1 cells were much lower than those from LNCaP cells. This is in line with a recent report [42]. PSA expression was up-regulated by DHT in both cell lines, although a higher concentration (100 times) was needed for 22Rv1 cells. RB346 alone did not stimulate the PSA secretion, but counterbalanced dose dependently the DHT induction (Fig. 4C and D). These results were confirmed when the whole cell extracts were considered (Fig. 4E). Both RB346 and bicalutamide inhibited transcription significantly (Fig. 4F).

3.7. Translocation

The cellular localization of the AR in the presence and absence of ligands was investigated using GFP-chimerical constructs (Fig. 5). In the absence of DHT, both WT-AR and T877A-AR were distributed in the cytoplasm, with a stronger proportion of receptor within the nucleus, when T877A-AR was over expressed, an observation described previously [43]. Upon treatment with 10 μM RB346 for 1 h, all four types of ARs significantly translocated into the nucleus. Similar results were previously reported for bicalutamide [43]. These experiments further demonstrated that the RB346 interacts with the WT-AR and also with T877A-, W741C- and W741L-AR and suggested that the antagonistic properties of RB346 are likely due to conformational changes of the AR in direction of recruiting coactivators, rather than impairing nuclear translocation.

3.8. Induced fit docking

To begin to understand how RB346 interacts with WT-AR and its mutants, we performed docking studies with Gide SP. Since the RB346 compound was larger than any previously modeled steroidal androgen or antiandrogen, flexible receptor studies were employed using Schrodinger's Induced Fit Protocol (IFD) [32]. IFD allows for prediction of how various AR agonists and antagonists bind the LBD. For validation of the method, docking of the co-crystallized ligand, or "self-docking", was performed for each of the three X-ray crystallographic structures. As CPA is the only co-crystallized steroidal antiandrogen with a bulky substituent at the C17 position, it was also docked into 1i37 and 1z95 and compared with 2oz7 (Supplementary Table S2). This was done to validate the prediction of a larger ligand being docked into conformationally different binding pockets. Root mean square deviations (r.m.s.d.) lower than 2.0 Å were observed in every case indicating that the ligands were correctly docked (Table S2).

With success in self-docking for all three AR mutants as well as validated predictions of CPA, RB346 was docked into WT (1i37) and mutant receptors T877A (2oz7) and W741L (1z95). Best scoring docking predictions for each receptor were compared to co-crystallized complexes (Fig. 6B–D). Results showed a similar binding mode of RB346 to CPA, with the side chain arm pointing towards the intersection of helices 3, 6, 7 and 11 (Fig. 6).

Although some variation in the side chain was seen for the WT and the mutant ARs, this is to be expected in such a flexible binding site, with a molecule with so many degrees of freedom, and with different point mutations giving alternative binding site volumes.

Key hydrogen binding interactions were preserved between RB346 and Arg-752, Gln-711, Asn-705 or Thr-877. The side chain of RB346 is proximal to hydrophobic residues, gaining favorable non-polar interactions in all three cases. Additionally making hydrogen bonds with the carbamate were observed. The plane of the steroid core rotates, likely due to the different position and side-chains at residue 741.

4. Discussion

The present study reports the screening of several DHT derivatives and the identification of a potential candidate that antagonizes in a significant manner without any agonistic property the WT-AR and clinically relevant AR mutants T877A, W741C, W741L and H874Y. The investigation therefore supports our hypothesis that DHT-derivatives such as RB346 have the potential to be efficient AR antagonists.

To the best of our knowledge this is the first DHT derivative described so far with a potent antiandrogen activity. The previous DHT derivative molecules reported, possessing one bulky substituent at C18 atom, turned out to be potent agonists of the AR [44]. Ligand binding needs to induce a specific conformation of several helices in ARs for agonist activity (Fig. 6A). First, the Helix 12 (H12) folding, subsequent to the agonist binding, is a prerequisite for AR agonistic function. Second, a proper hydrophobic binding surface composed of H3, H4, and holo-H12 has to be generated in order to recruit coactivators. Destabilization of one of those events leads to antagonistic activity. Molecules with bulky side chains preventing H12 helix from adopting its active conformation are called "direct" antagonists. This type of antagonism is observed mainly with fulvestrant and 4-hydroxytamoxifen for ER [45] and is probably also involved with MDV3100 for AR [28]. In contrast "indirect" antagonists do not prevent H12 from adopting an active conformation and instead stabilizes nonproductive conformations of key binding site residues. Shiau and colleagues established the concept of "indirect antagonism" first called "passive antagonist" [46]. Molecular modeling (Fig. 6) indicates that the bulky side chain of RB346 does not affect H12 folding. By analyzing the binding mode of RB346 with WT and mutant ARs we found that likely RB346 remained antagonistic probably by modifying its anchorage accordingly with the various LBD. With the predicted orientation of the RB346 side chain pointing towards the end of H11, the W741L mutation, conferring agonist activity to other known antagonists such as R-bicalutamide and hydroxyflutamide, would not affect RB346. Interestingly,

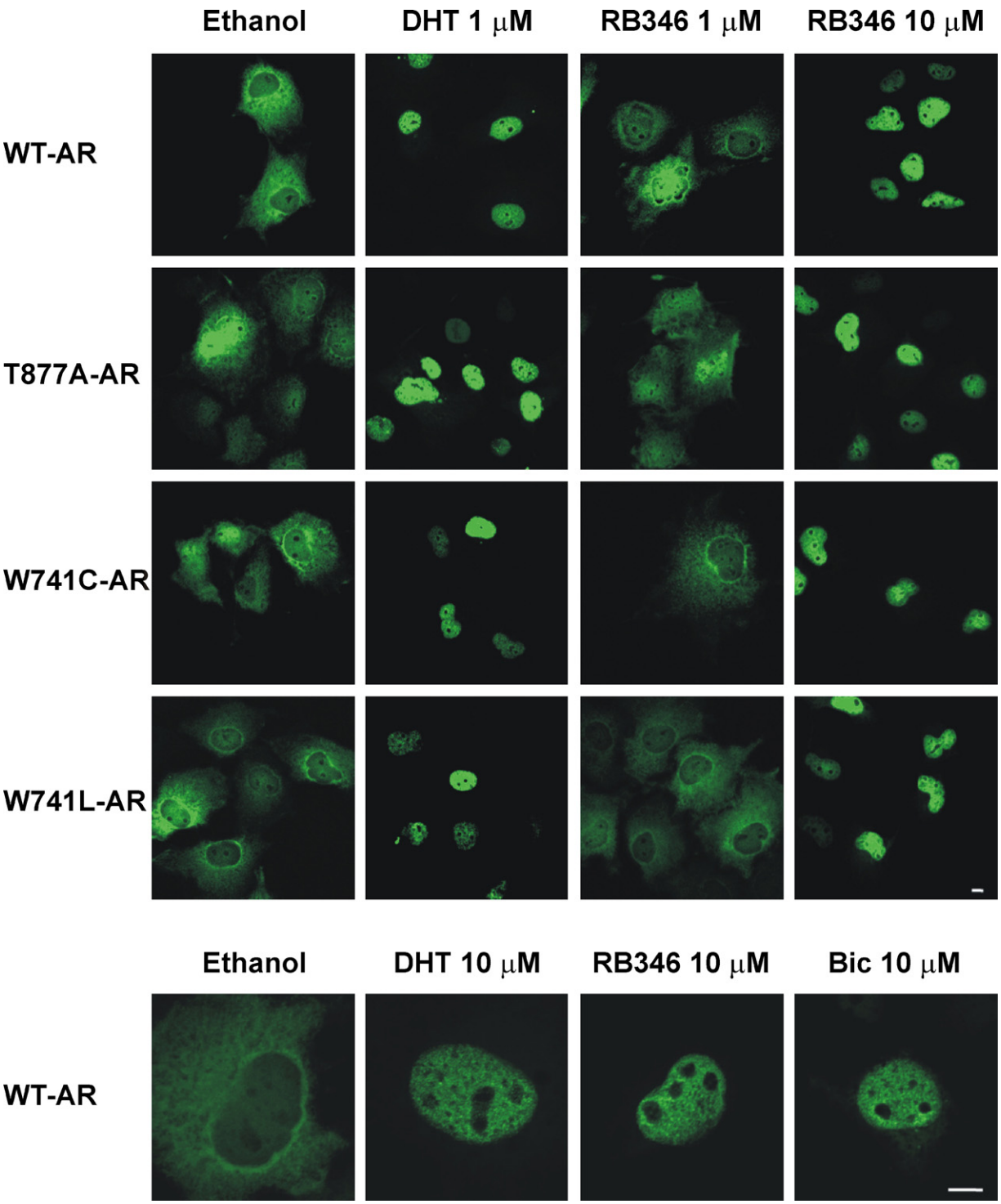

Fig. 5. Translocation assays. COS-7 cells transfected with pSG5-WT-AR-GFP, pSG5-T87A-AR-GFP, pSG5-W741C-AR-GFP or pSG5-W741L-AR-GFP were treated 24 h later with DHT (1 μM), bicalutamide (10 μM), RB346 (1–10 μM) for 1 h (scale bars, 5 μm).

MDV3100 and RB346 antagonize a broad range of mutated ARs whereas they harbor a complete different structure and work through different mechanisms.

Interestingly at 1 μM RB346 demonstrated antagonistic efficacy (Fig. 2A, B) by binding to the AR (Fig. 2C), but the bound AR-RB346 complex did not translocate to the nucleus (Fig. 5). This phenomenon resembles the action of MDV3100 [28]. Importantly, at 10 μM RB346 presented both antagonistic efficacy (Fig. 2A, B) and nuclear translocation (Fig. 5). This suggests that the sequestration of AR in the cytoplasm observed at 1 μM is probably not the main antagonistic mechanism of RB346. In the presence of high concentration of RB346 (10 μM) foci were observed in the nucleus but no agonistic activity was encountered, which suggests that the RB346 might induce DNA binding to the DNA of AR. These observations together with the modeling prediction support the hypothesis that RB346 induces a unique conformation in the AR causing nuclear translocation, binding to androgen responsive elements (AREs) and recruitment of nuclear receptor corepressors, a mechanism involved for the GR antagonist mifepristone [47] or for bicalutamide [48,49].

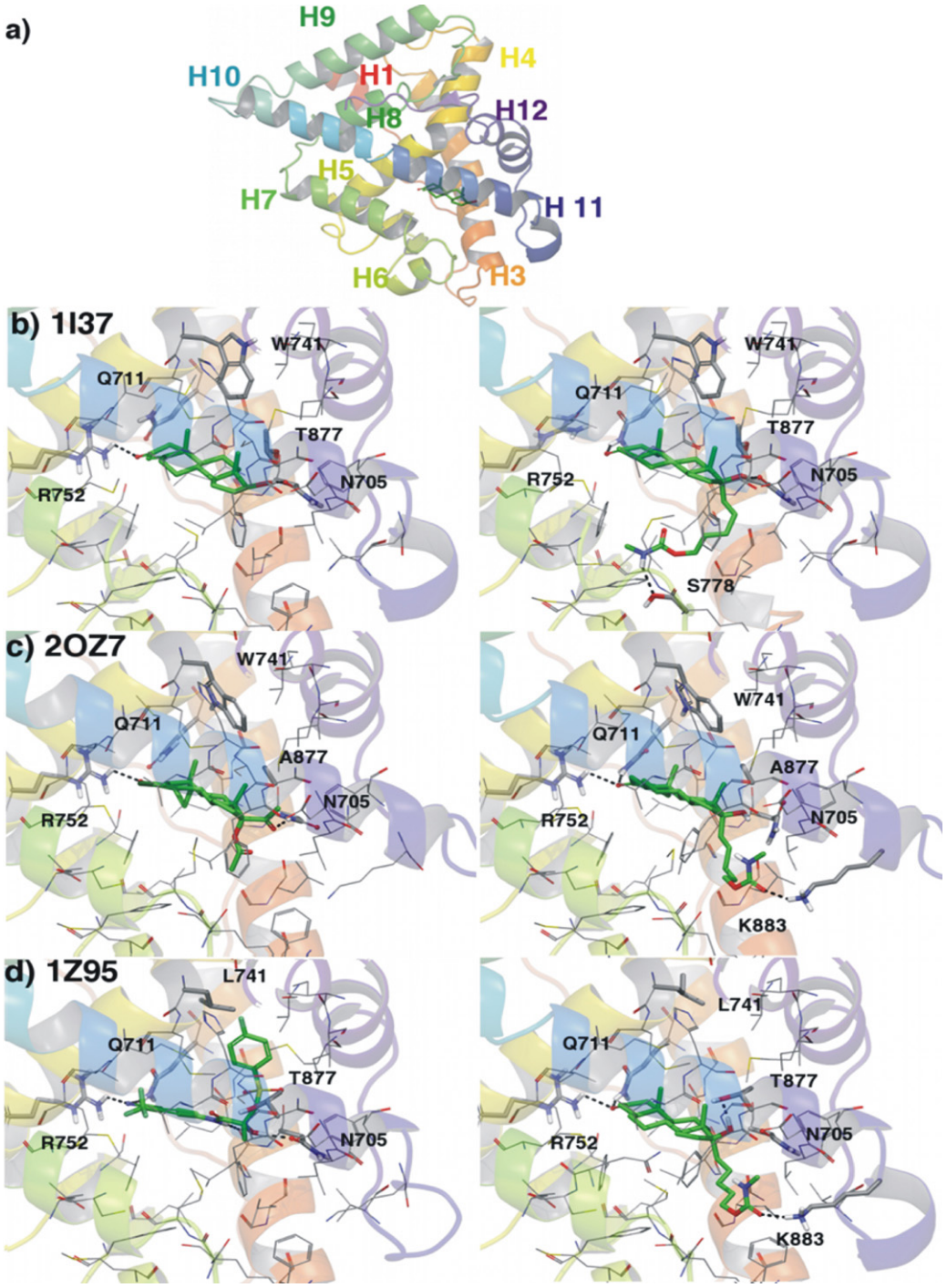

Fig. 6. Induced fit docking for RB346 accommodation. Cartoon representation of overall fold of AR colored by residue position and labeled by helix (A), with insight views for each three AR mutant co-crystal structures (left) and RB346 docked (right); (B) ligand binding domain of WT-AR with DHT; (C) ligand binding domain of T877A-AR with CPA; (D) ligand binding domain of W741L-AR with R-bicalutamide.

Steroidal antagonists described until now were mainly progesterone derivatives harboring PR agonistic function (rev in [3]). The simultaneous modulation of AR and PR activity by steroidal compounds may be beneficial. As an example CPA interferes with the AR and also suppresses GnRH and LH secretion due to its PR agonist activity. This leads to a decrease of testosterone secretion from the testis, a desired effect for the androgen depletion therapy but less so for potency (rev by [50]). In contrast bicalutamide lacks progestational property, antagonizes only the AR and causes an increase of LH and testosterone levels in serum. As a consequence bicalutamide preserves libido and potency in approximately 80% of the patients [51–53]. For this reason nonsteroidal antiandrogens were preferentially developed. In contrast to the progesterone derived antiandrogens, the antiandrogen RB346 harbors moderate anti-progesterone activity. Therefore it is reasonable to speculate that RB346 will not down-regulate testosterone levels in the serum and preserve potency as bicalutamide does.

Interestingly, replacement of the 3 keto group of a pregnenolone derivative by a 3β-hydroxyl group participates in the AR selectivity [54]. Whether or not 3β-hydroxyl substituted DHT derivative would keep their antagonist function for mutants such as W741C is not yet known and additional studies need to be

performed in order to characterize the structural requirement for selectivity towards AR or PR.

An important issue of a potential therapeutic agent is its stability and the kinetics. For this purpose we did an in silico analysis of RB346. The in silico ADME (absorption, distribution, metabolism, and excretion) profile was characterized using QikProp software (version 2.4; Schrodinger). The analysis predicted favorable properties for RB346. The compound presented apparently good cell permeability and was stated as an orally active drug without violation of any of the 5 Lipinski's rules (Supplementary Table S3). The compound has low water solubility, a potentially modifiable property, which induced a violation of one of the 3 Jorgensen's rules. It is important to remember that both rules do not predict whether the compound is pharmacologically active. The predictions rely solely on molecular properties that ensure biodisponibility for that particular compound. Additionally, the stability of the compound RB346 was assessed in vitro. The compound was incubated in human plasma and the amount quantified by thin layer chromatography and GC–MS. During the 24 h incubation time only 20% of the parental compound was degraded, an important prerequisite for biological efficacy in vitro.

Since the AR is expressed in skeletal muscle, liver, skin and central nervous system, with the highest expression observed in the prostate, adrenal gland and epididymis (rev by [55]), it is not surprising that androgen deprivation therapy induces changes in the body composition (rev by [56]). Additionally, it is noteworthy that in the prostate itself AR has a dual function, as a stimulator or survival factor and as well as a suppressor of cancer progression and metastasis depending on the prostatic cell type (rev by [57]). Thus, there is a clear need for targeting the delivery of antiandrogens to minimize side effects. A conjugate involving bicalutamide, a nuclear localization signal and carrier peptide has shown some specific effects by inhibiting LNCaP cell proliferation [58]. For such specific applications steroidal antiandrogens without agonistic effect such as RB346 antagonizing WT and main AR mutants, in contrast to CPA or bicalutamide, have much promise.

In conclusion, we report the design and the test of a DHT derived antagonist. In contrast to CPA and to bicalutamide, RB346 antagonizes the main mutants of the AR and inhibits growth of androgen sensitive prostate cells. One unique characteristic of RB346 is the flexibility of its side chain which has the property to adapt itself to the different mutated LBDs. This is of significance as it could theoretically delay in some extent hormone refractory PCa occurrence. In this respect, the study supports the identification of a novel antiandrogen.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.08.020.

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