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Stable Expression of the Human 5α-Reductase Isoenzymes Type I and Type II in HEK293 Cells to Identify Dual and Selective Inhibitors

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A eucaryotic cell assay was established to identify novel, dual and selective inhibitors of human 5α-reductase. For this purpose the cDNAs encoding 5α-reductase type I and type II were inserted into a pRcCMV vector and expressed in human embryonic kidney (HEK293) cells. Single cell clones with substantially high enzymatic activity were selected and established as permanent cell lines. K_M values were determined for both isozymes. The inhibitory potency of several steroidal and non-steroidal compounds synthesized in our group, as well as finasteride and 4MA as controls, were tested by measuring the conversion of [^{3}H]androstenedione. Reaction products were quantified by a HPLC reversed phase technique. Using the new cell assays, selective as well as novel dual 5α-reductase inhibitors with IC_{50} values between 1.0 and 2.5 μM were identified.

Keywords: Human 5α-reductase, HEK293 cells, dual inhibitors, selective inhibitors, finasteride

INTRODUCTION

NADPH-dependent steroid 5α-reductase catalyzes the formation of the most active androgen dihydrotestosterone (DHT) from testosterone (T). As DHT plays a predominant role in the development of prostate cancer,[1] benign prostatic hyperplasia (BPH)[2] and other disorders, such as acne,[3] female hirsutism[4] and male pattern baldness,[5] 5α-reductase is an important therapeutic target. In general, the enzyme is able to reduce the 4, 5-double bond of a 3-oxo-4-ene to the corresponding 5α-3-oxo derivative and therefore also converts androstenedione (AD) to androstanedione (DHAD).[6]

Two different isoforms of steroid 5α-reductase have been identified, chronologically termed type I and type II.[7,8] The isozymes are encoded by separate genes, which are expressed differentially in human tissues. Whereas type II is predominantly expressed in the prostate, 5α-reductase type I is mainly formed in the scalp skin. Both enzymes, however, can be found at lower levels in other tissue e.g. in the liver. The distinct distribution of both isoforms offers the possibility of selective inhibition of the type I isoform for acne or hirsutism and inhibition of

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type II for BPH or prostate cancer. For the latter disease decreasing the overall DHT level by dual inhibitors of both 5α-reductases is regarded to be even more promising.[9]

A wide variety of non-steroidal 5α-reductase inhibitors have been synthesized in our group.[10–12] Because of limited availability of human tissue e.g. prostate, the aim of this study was to provide a recombinant and rapid test assay to evaluate these compounds regarding their dual or selective inhibitory potential.

The recombinant approach was chosen as we wanted to measure both isozymes individually with an identical cellular background. For this purpose human embryonic kidney cell line HEK293, which lacks endogenous 5α-reductase activity was transfected with the cDNA for either of the isoforms. Stable clones were selected, tested on enzyme activity and established as permanent cell lines. The cell lines were used to test selected compounds of our group as well as the steroidal inhibitors finasteride and 4MA as controls. Using this strategy dual and selective inhibitors of both 5α-reductase isozymes could be identified.

MATERIALS AND METHODS

Cell Culture

The adherent fibroblastoid HEK293 cell line was obtained from DSMZ, Braunschweig, Germany (DSM ACC 305) and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 0.25% sodium hydrogen carbonate, 100 units penicillin/ml, and 100 μg streptomycin/ml. The cells were grown in a humidified 95% O_2-5% CO_2 atmosphere at 37 °C in 175 cm^2 tissue culture flasks (Nunc, Wiesbaden, Germany). Every 3–4 days they were split at a ratio of 1:6. For transfection experiments cells were used at passage number 8–10. Tissue culture reagents were from c.c.pro (Neustadt/W., Germany), except G418 sulfate, which was from Calbiochem (Bad Soden, Germany).

Construction of 5α-Reductase Expression Plasmids

The *Not*I-insert of the plasmid ph5α45,[8] which was kindly provided by D. W. Russell, is a full length human cDNA encoding the type I 5α-reductase isoenzyme. It was inserted downstream of the Cytomegalovirus (CMV) promotor of the eucaryotic expression vector pRcCMV (Invitrogen, Groningen, Netherlands). This vector carries an additional neomycin resistance. The new construct which encoded human 5α-reductase type I was named pRcCMV-I and used for transfection.

The *Sal*I/*Not*I-insert of the plasmid pBS-76–1[7] (provided by D. W. Russell) corresponds to the full length human cDNA encoding the 5α-reductase type II isoenzyme. It was first inserted by the *Sal*I/*Not*I-sites into pUC21-vector[18] and recleaved by *Hin*dIII and *Xba*I. By this strategy a 5′-*Hin*dIII-and a 3′-*Xba*I-site was added to the 5α-reductase type II encoding DNA fragment, by which it was inserted into the expression vector pRcCMV. The resulting plasmid (pRcCMV-II) was used for transfecting HEK293 cells.

Transfection Procedure

One day before the transfection experiment 1×10^7 HEK293 cells were seeded in 100 mm culture dishes (Nunc, Wiesbaden, Germany). By this procedure the culture will be approximately 70% confluent on the day of transfection. The liposomal transfecting reagent Roti®-Fect (Roth, Karlsruhe, Germany) was used for transfecting cells either with pRcCMV-I or pRcCMV-II following the manufacturers recommendations. The optimal DNA/reagent ratio was 10 μg plasmid and 20 μl Roti®-Fect reagent.

Selection of Stable Clones

Initially the concentration at which G418 sulfate inhibits the growth of untransfected HEK293

cells was determined. Therefore varying concentrations of G418 sulfate (50 µg/ml–1000 µg/ml) were added to adherent HEK293 cells seeded in 24-well tissue culture plates at a density of 200,000 cells/well. After 6 days incubation at 37 °C, viable cells were determined using the trypan blue exclusion test. At a dose of 400 µg/ml G418 sulfate 50% of the cells were killed.

Two days after transfection the growth medium was replaced by medium containing 500 µg/ml G418 sulfate. During the following 3 weeks of incubation untransfected cells subsequently died. To remove cell debris the medium was replaced every four days. Stable cell clones could be identified by phase contrast microscopy at the end of the second week. Single cell clones were picked and transferred into 60 mm culture plates for further analysis.

Inhibition Assay

HEK293 cells (300,000/well) transfected either with pRcCMV-I or with pRcCMV-II were seeded in a 24-well tissue culture plate (Nunc, Wiesbaden, Germany) and incubated overnight to allow attachment of the cells. The medium was removed by aspiration and replaced by 0.5 ml of a freshly prepared substrate/inhibitor solution. This solution consisted of complete DMEM-medium with 5 nM [^{3}H] androstenedione (NEN Dupont, Köln, Germany) and 1% of an adequate dilution of the inhibitor. Inhibitors were dissolved in absolute dimethyl sulfoxide (DMSO) and a 10 µM final concentration was used for initial experiments. Controls were performed with 5 µl DMSO (without inhibitor) and 495 µl DMEM containing the substrate. After the specified incubation periods 500 µl supernatant was removed from the cells and the steroids were extracted with 800 µl diethylether. The organic phase was transferred and evaporated. The dried steroids were resuspended in 50 µl methanol and subjected to HPLC analysis.

Reversed Phase HPLC

HPLC analyses were performed by the use of a high pressure solvent delivery pump (Waters M6000A, Milford, USA), a radioactivity detector (LB506C, Berthold, Wildbad, Germany) and an autosampler system (851-AS, Jasco, Tokyo, Japan). Nucleosil 120–3-C_8 was applied as stationary phase using prepacked columns (125×4 mm; Macherey-Nagel, Düren, Germany). The injection volume was 13 µl and methanol/water (50/50) was used as the mobile phase for separation of the steroid metabolites. Applying a flow rate of 0.4 ml/min, the retention times were as follows: androstenedione: 11.2 min, androstanedione: 17.5 min. Data acquisition and integration was carried out by the use of the HALABE 1.6.5 software (Berthold, Wildbad, Germany).

Inhibitors

5α-reductase inhibitors used in this study are shown in Figure 1. Finasteride and 4MA were kindly provided by Merck Sharp & Dohme (Rahway, USA). All other compounds were synthesized in our group.

RESULTS AND DISCUSSION

HEK293 Cells Expressing 5α-Reductase Isozyme Type I

After transfection of HEK293 cells with the expression plasmid pRcCMV-I eight G418-resistant clones could be identified, which exhibited 5α-reductase activity. During a 20 minute incubation period, 50% of the substrate (AD) was converted by a 80% confluent cell culture in 60 mm culture dishes. All single cell clones were frozen in liquid nitrogen. For all clones 5α-reductase activity remained stable after thawing and recultivation. Only one of this 5α-reductase type I expressing clones was selected for further investigations and referred to as

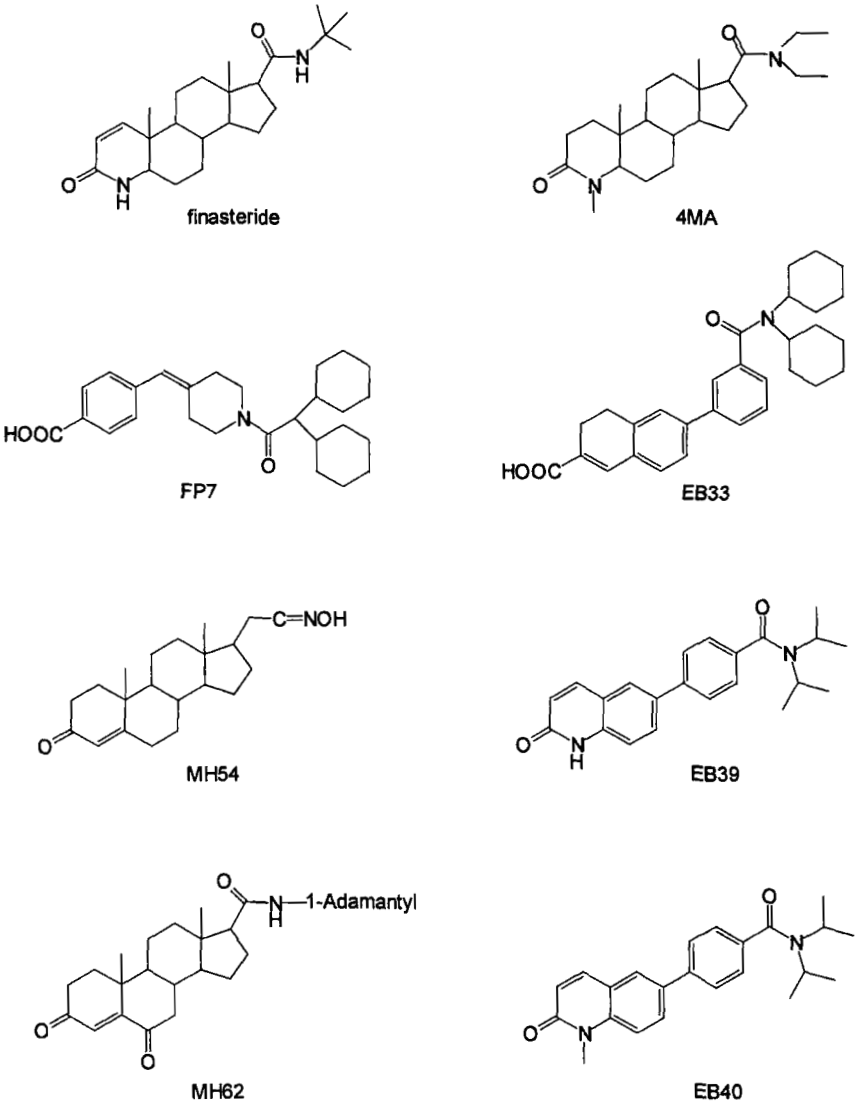

FIGURE 1 Inhibitors of 5α-reductase.

HEK293-5αI. An 80% confluent culture of these cells in 24-well plates exhibited constant enzymatic activity during an incubation period of one hour (Figure 2). Almost 20% conversion of the substrate AD to the product DHAD was achieved after 30 minutes. This timepoint was chosen for the assay. During incubation no testosterone formation due to an internal 17β-hydroxysteroid dehydrogenase activity of the cells could be detected by HPLC analysis.

HEK293 Cells Expressing 5α-Reductase *Isozyme Type II*

The selection of G418-resistant HEK293 cells which had been transfected with the expression plasmid pRcCMV-II yielded 14 different single cell clones. All of them expressed 5α-reductase activity in the same high level range. During a 20 minutes incubation period about 50–70% of the substrate AD was converted to DHAD by an

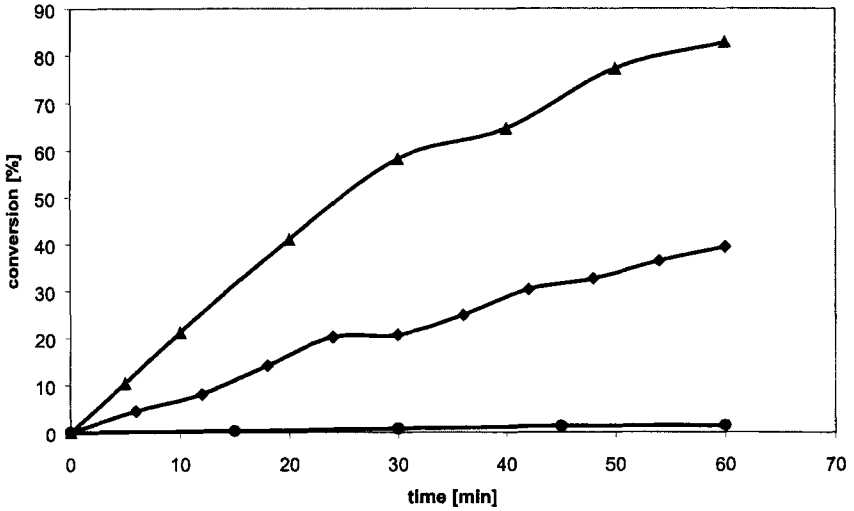

FIGURE 2 [^{3}H]androstenedione conversion rates of HEK293-5αI (♦) and HEK293-5αII (▲) as well as substrate conversion by untransfected HEK293 cells (●). Each value was determined in duplicate using 80% confluent cell cultures in 24-well plates.

80% confluent culture in 60 mm culture dishes. Cells could be frozen and recultivated without measurable reduction of enzymatic activity. One clone was selected for further experiments and termed HEK293-5αII. A time-dependent substrate conversion curve was obtained (Figure 2). 20% conversion of the substrate occurred after 13 min incubation.

Comparative Expression Studies

As can be seen in Figure 2, no endogenous background of 5α-reductase activity could be observed in untransfected HEK293 cells. Although HEK293-5αI and HEK293-5αII cells were both transfected with plasmids based on the same vector system including the strong cytomegalovirus promotor (P_{CMV}), the obtained 5α-reductase activities were different. HEK293 cells expressing the type II isoform exhibited a more than twofold higher enzymatic activity than type I expressing HEK293 cells. A possible explanation can be, that the human cDNA fragment which was used to construct the expression vector pRcCMV-I contains an additional ATG start codon (Figure 3). This additional upstream start codon may result in the production of misfolded variants or reduced amounts of active 5α-reductase by spurious translation initiation.[13] Within the coding sequence of 5α-reductase type II, as part of the plasmid pRcCMV-II, a similar codon is not present.

K_M-Values for 5α-Reductase Isozyme Expressed by HEK293 Cells

K_M-values were determined for both enzymes in the whole cell assay by Lineweaver-Burk plot analysis, AD concentrations in the range from 0.05–2000 μM being used. The obtained substrate affinities (K_M type I = 1.59 μM and K_M type II = 0.56 μM) were of the same order of magnitude as those obtained earlier with both isozymes expressed in CHO cells.[14]

Inhibition Studies

HEK293–5αI cells and HEK293–5αII cells were used to test finasteride and 4MA, well known steroidal inhibitors of 5α-reductase as well as two selected steroidal and four selected nonsteroidal compounds of our group. In Figure 4, the inhibition values at concentration of 10 μM are shown. All compounds had a considerable

Type I:	G GGC ***ATG*** GAG CAC GCT GCC CAG CCG **ATG**
Type II:	GCG GCC ACC GGC GAG GAA CAC GGC GCG **ATG**

FIGURE 3 DNA sequences located upstream of the coding regions of 5α-reductase isoenzymes type I and type II. Regular start codons are in bold letters, additional start codon in type I encoding DNA sequence is in italics.

inhibitory effect on at least one isoform. Finasteride and 4 MA inhibited both enzymes completely, MH54 and MH62 showed very strong inhibitory effects and moderate inhibition of both isoforms was observed for EB33. EB39 is a rather selective compound inhibiting the type II enzyme stronger than the type I isoform. EB40 and FP7 are highly active and selective inhibitors of type I and type II enzyme, respectively. IC_{50} values were determined for compounds which inhibited one isoform more than 70% (Table I). 4MA is a very potent inhibitor of both isoforms. Finasteride shows a tenfold stronger inhibition of the type II enzyme. These data are similar to those obtained earlier with non-human recombinant expression systems[14–16] or human non-whole cell assays[17] and confirm that finasteride is a selective type II inhibitor. Although they are less active than finasteride and 4MA, the novel compounds tested here, especially FP7 and EB40, are promising steps towards selective inhibition of either type I or type II 5α-reductase.

The present paper shows that the new established cell lines HEK293-5αI and HEK293-5αII are appropriate to easily identify selective and dual inhibitors of 5α-reductase type I and type II. They can be freeze-stored and recultivated, thus enabling easy and reproducible testing. Compared to the use of microsomal enzymes, whole cell systems are closer to the *in vivo* situation. The fact that human enzymes have been expressed makes these tools real patient-oriented test systems.

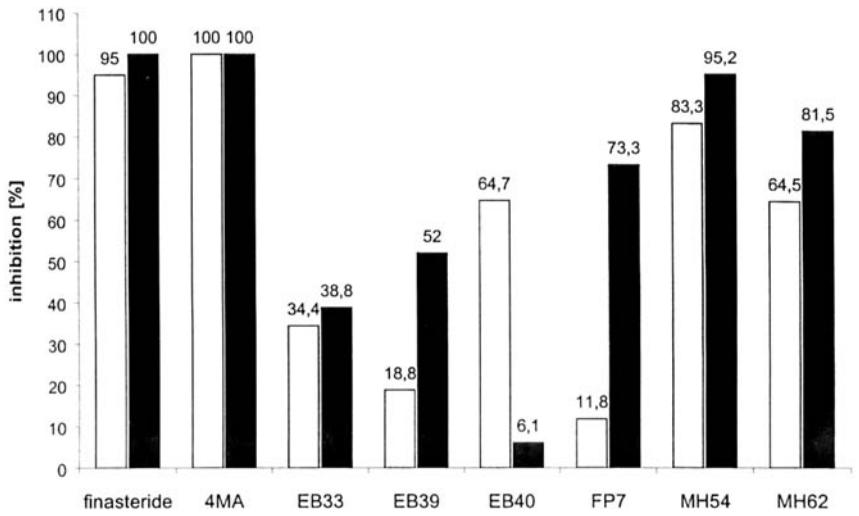

FIGURE 4 Inhibition of 5α-reductase type I (white) and type II (black) activity, expressed in HEK293 cells, by different compounds at 10 μM concentration.

TABLE I IC_{50} values of different compounds on 5α-reductase type I and type II expressed in HEK293 cells

compound	5α-reductase type I IC_{50} [μM]	5α-reductase type II IC_{50} [μM]
4MA	0.015	0.075
finasteride	0.55	0.057
FP7	—*	1.29
MH54	2.44	0.97
MH62	—*	2.34

*Inhibition less than 70% at 10 μM concentration.

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