5α-Reductase in Intact DU145 Cells: Evidence for Isozyme I and Evaluation of Novel Inhibitors☆

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Summary

The epithelial-like human prostatic carcinoma cell line DU145, which expresses 5α-reductase type I isozyme, was used to test a series of potential 5α-reductase inhibitors. The exclusive expression of the type I isozyme was confirmed by PCR and subsequent DNA sequence analysis. Culture conditions were optimized for high conversion rates. Using this whole cell assay finasteride, 4MA, and 65 steroidal and non-steroidal compounds synthesized in our group were tested for their inhibitory activity. Inhibitors with IC_{50} values in the nanomolar range could be identified.

Introduction

Excessive androgen action in the skin causes disorders such as male pattern baldness [1], female hirsutism [2], acne, and seborrhoea [3]. The most potent androgen dihydrotestosterone (DHT) is synthesized by 5α-reductase [4] which exists in two isoforms [5, 6]. Whereas the type II enzyme is the main isoform expressed in the prostate, the type I isozyme is present in the nongenital skin and hair follicles [7, 8]. Selective inhibition of 5α-reductase type I can reduce high androgen levels in the skin and might therefore be a promising strategy for the treatment of androgen-dependent dermatological disorders. To identify selective inhibitors an assay is needed for the evaluation of 5α-reductase type I inhibition. The epithelial-like human prostatic carcinoma cell line DU145 has been described to express exclusively the 5α-reductase type I isoform [9, 10]. In the present paper this was confirmed by PCR and sequence analysis. DU145 cells were used to establish a 5α-reductase type I assay. Using this assay, a large variety of steroidal and non-steroidal compounds were tested and highly potent inhibitors could be identified.

Results and Discussion

PCR and Sequence Analysis of DU145 5α-Reductase

DU145 has been described to express 5α-reductase type I. This was confirmed in earlier studies by northern blot analysis [10]. At first sight, however, this seemed to be inconsistent with the origin of DU145, being a brain metastasis from prostate cancer. To verify the type I character of DU145 5α-reductase, PCR was performed. For this purpose a type I specific primer pair (WOR1/WOR2) was designed and synthesized according to the published DNA sequence [5], as well as a type II specific primer pair (WOR6/WOR7) [6]. As shown in Figure 1 only the use of the type I specific oligonucleotides WOR1 and WOR2 yielded an amplified fragment in DU145 cells, whereas PCR with WOR6 and WOR7 was negative. The size of the obtained fragment corresponds to that predicted from type I 5α-reductase cDNA sequence (884 bp) and was identical with that obtained in the control PCR using the type I encoding plasmid ph5α45 [5] as a template.

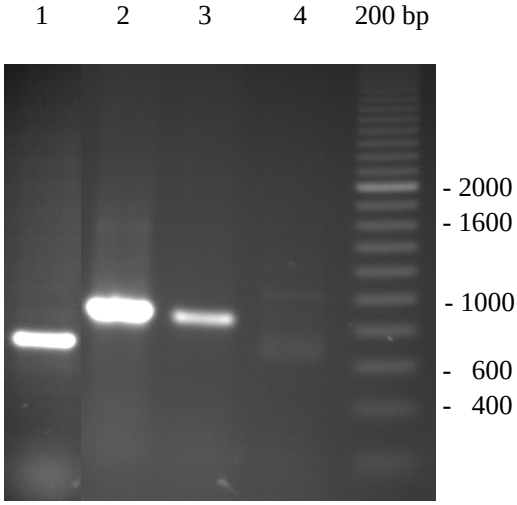

Figure 1. PCR with 5α-reductase type I specific primers WOR1/WOR2 (lanes 2, 3) and 5α-reductase type II specific primers WOR6/WOR7 (lanes 1, 4). Templates were pBS-76-1 (lane 1), ph5α45 (2), and DU145 (3, 4). A 1.5% agarose gel stained with ethidium bromide is shown.

Whereas PCR with WOR6/WOR7 using the type II encoding plasmid pBS-76-1 (control) [6] yielded a DNA fragment of the predicted size (774 bp), no fragment was obtained with DU145 using this primer combination. To exclude that this was caused by PCR conditions, a temperature gradient PCR was performed in the range of 46.6–66.0 °C annealing temperature. In DU145 cells a fragment was only obtained with WOR1/WOR2 but not with WOR6/WOR7 (not shown). Neither the cross combination WOR1/WOR7 nor WOR6/WOR2 resulted in a PCR fragment amplification. For

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examination of eventual amino acid exchanges, the PCR fragment obtained with primer pair WOR1/WOR2 (Fig. 1, lane 3) was cloned and subjected to DNA sequence analysis. The resulting DNA sequence was identical with that of 5α-reductase type I published by Andersson and Russell [5]. No nucleotide exchange and consequently no amino acid exchange could be detected.

From these results it can be concluded that DU145 expresses only 5α-reductase type I with no mutation or amino acid exchange, respectively. This findings can be easily understood taking into account recent results. Northern blot analysis and RT-PCR studies with isolated stromal and epithelial cells of human prostates revealed that isozyme I is expressed in epithelial and isozyme II in stromal cells [11, 12]. From this it can be concluded that DU145 cells originated from an epithelial cancer.

Establishment of Assay Procedure

Different assay conditions were investigated to establish a DU145 assay. The cell number plated out per well and the substrate incubation time were varied. As seen in Figure 2 conversion of 5 nM [^{3}H]androstenedione to [^{3}H]androstanedione was linear within a period of 10 hours. Even for high cell numbers substrate concentration was sufficient. For further experiments 160,000 cells were plated out. An incubation time of 6 hours was chosen (substrate conversion rate below 25%, Figure 2).

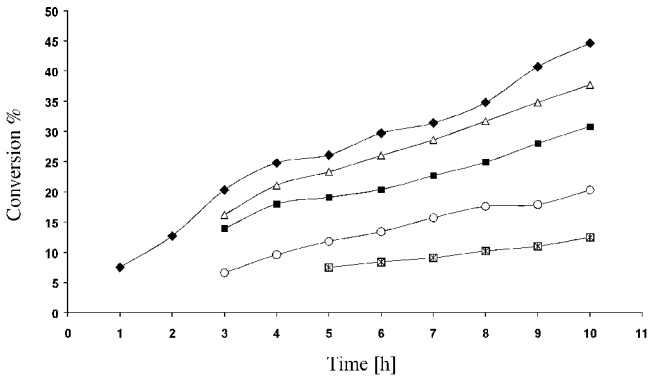

Figure 2. [^{3}H]Androstenedione conversion rates in DU145 cells at different time points; graphs represent cell numbers from 40,000 (bottom) to 200,000 (top) cells per 24-well.

Inhibition of DU145 5α-Reductase by Finasteride, 4MA, and Novel Compounds

Finasteride and 4MA (Figure 3) are well known inhibitors of human 5α-reductase type I. The IC_{50} values determined in the prostate cancer cell line HPC-36M as well as in COS cells expressing human type I isozyme revealed that 4MA is at least ten times more potent than finasteride [5, 13]. As seen in Table 1, the IC_{50} values obtained in the DU145 whole cell assay are of the same order of magnitude. Moreover the determined IC_{50} value for finasteride is identical to that determined by Guarna et al. [14].

This assay was used to test 65 potential inhibitors of 5α-reductase type II synthesized in our group. Sixteen compounds exhibited an inhibition higher than 75% at a concentration of 10 μM. These were chosen for IC_{50} value determination. The structures of the most potent inhibitors with IC_{50} values below 1.5 μM are given in Figure 4. As shown in Table 1, the non-steroidal compounds EB33, EB34, EB40, and MK77 as well as the steroidal compound MH65 exhibited IC_{50} values in the nanomolar range.

Using a whole cell assay, only compounds exhibit inhibitory activity which are able to penetrate the cell membrane, which is a prerequisite for in vivo activity. Some of the identified novel compounds might be leads for the development of 5α-reductase isozyme I inhibitors.

Table 1. Inhibition of 5α-reductase type I in DU145: IC_{50} values of finasteride, 4MA, and novel inhibitors.

Compound	IC_{50} value [μM]
Finasteride	0.043 ± 0.005
4MA	0.006 ± 0.001
EB18	1.42 ± 0.13
EB25	1.23 ± 0.11
EB33	0.81 ± 0.06
EB34	0.68 ± 0.12
EB40	0.51 ± 0.21
MK77	0.46 ± 0.14
MH65	0.92 ± 0.03

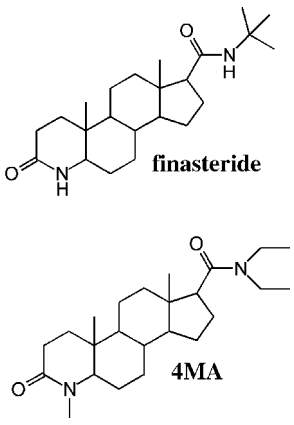

Figure 3. Structure of the steroidal 5α-reductase inhibitors finasteride and 4MA.

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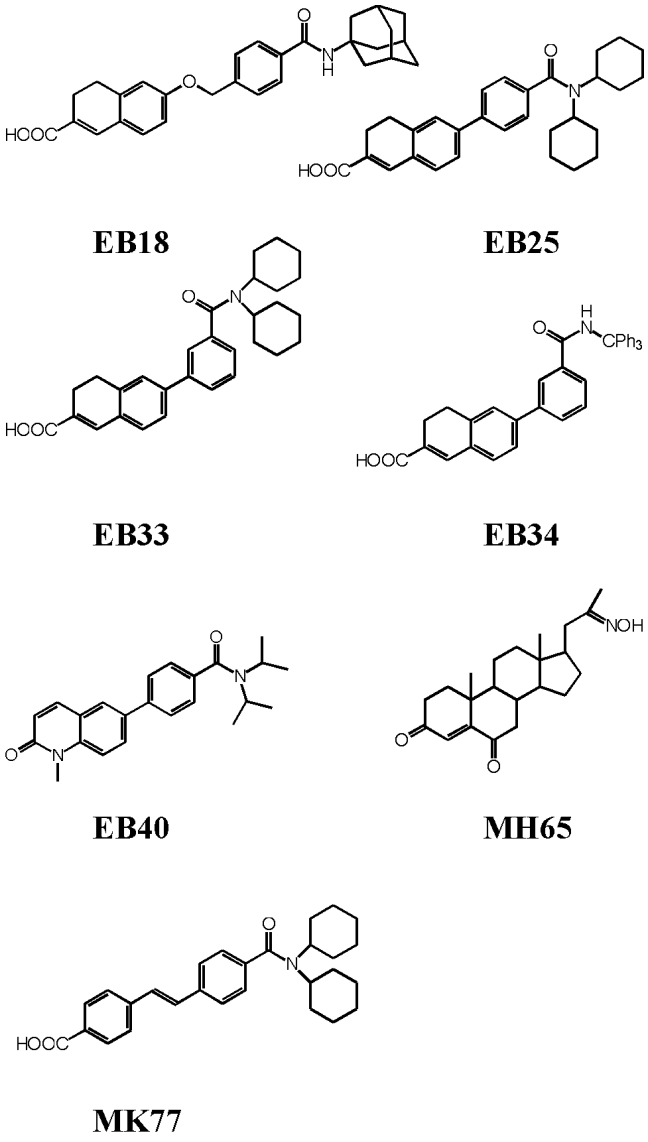

Figure 4. Structures of new 5α-reductase type I inhibitors identified using the DU145 whole cell assay.

Experimental

Cell Culture

The human prostatic carcinoma cell line DU145 (DSM ACC 261) was obtained from the DSMZ (German collection of microorganisms and cell cultures, Braunschweig, Germany). The cells were maintained as a monolayer culture in RPMI-1640 medium. The medium was supplemented with 10% (*v/v*) fetal calf serum (FCS), 0.25% sodium bicarbonate, 100 units penicillin/ml and 100 μg streptomycin/ml. Cells were grown in 175 cm^2 tissue culture flasks (Nunc, Wiesbaden, Germany) in a humidified 95% O_2 and 5% CO_2 atmosphere at 37 °C. The cell line was routinely passaged every 4 days after treatment with 0.25% trypsin solution. All tissue culture reagents were obtained from c.c.pro (Neustadt/W., Germany).

PCR and Sequence Analysis

The following primers were used for PCR experiments: WOR1 (5′-CCC AAG CTT GGG ACC ATG GCA ACG GCG ACG GGG GTG GCG G-3′), WOR2 (5′-AAA GTC CAT AGA GAA GCG CCA TTG G-3′), WOR6 (5′-GAA CAC GGC GCG ATG CAG GTT CAG TGC CAG–3′) and WOR7 (5′-AAA AGA TGA ATG GAA TAA GGG CTT TCC G-3′). All primers were synthesized by MWG-Biotech (Ebersberg, Germany). PCR was performed with nucleic acid extracts from DU145 cells with or without RNase A treatment. PCR conditions (thermocycler primus 25, MWG-Biotech) were 1 min at 94 °C (denaturation), 2 min at 64 °C (anealing) and 3 min at 72 °C (elongation). As controls the plasmids ph5α45 and pBS-76-1 were used as templates. Plasmid ph5α45 contains a full-length human cDNA encoding 5α-reductase type I and plasmid pBS-76-1 contains a full-length human cDNA encoding 5α-reductase type II. Temperature gradient PCR was performed using a Mastercycler gradient apparatus (Eppendorf, Köln, Germany).

The PCR-amplified fragment encoding type I 5α-reductase was ligated into the pks (+)-cloning vector (Stratagene, Groningen, Netherlands) and submitted to DNA sequence analysis which was performed by SEQLAB (Göttingen, Germany).

Inhibition Assay

DU145 cells were used as source of 5α-reductase type I isozyme between passages 15–40. Cells were plated out one day before the experiment in 24-well plates at a density of 1.6×10^5 cells/well. DU145 cells were allowed to become adherent overnight in 1 ml RPMI-1640 medium (with 10% FCS). Appropriate concentrations (10 μM final concentration at initial tests) of inhibitors dissolved in dimethyl sulfoxide (DMSO) were applied in duplicates. Growth medium was replaced by 500 μl of fresh medium containing 5 μl of the inhibitor and 5 nM [^{3}H]androstenedione as substrate. At least 3 controls were performed with 5 μl DMSO (without inhibitor) and 495 μl RPMI (10% FCS) containing the substrate. After a 6 h incubation period (5% CO_2 in a humidified atmosphere at 37 °C), the medium was again replaced and extracted with 800 μl diethyl ether. Steroids were dried by evaporation of the organic phase. After resuspension in 50 μl ethanol steroid metabolites were subjected to HPLC analysis. Results are expressed as the amount of androstanedione produced as a percentage of control values. Compounds showing more than 70% inhibition of the substrate conversion rate (compared to the uninhibited controls) were subsequently tested at three different concentrations for the determination of IC_{50} values.

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 steroidal 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 were carried out by the use of the HALABE 1.6.5 software (Berthold, Wildbad, Germany).

Test Compounds

The new 5α-reductase inhibitors have been synthesized in our group: EB18, EB25, EB33 and EB34 [15], EB40 [16], MH65 [17], and MK77 [18].

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☆ Dedicated to Prof. H. Becker on the occasion of his 60th birthday.

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