Inhibition of 5α-reductase and aromatase by PHL-00801 (Prostatonin®), a combination of PY 102 (Pygeum africanum) and UR 102 (Urtica dioica) extracts

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Summary

This study was undertaken to evaluate a possible effect of the extracts PY 102 of Pygeum africanum (Hook), and UR 102 of Urtica dioica L. as well as their combination PHL-00801 (Prostatonin®) on the enzymes 5α-reductase (5α-RE) and aromatase (AR):

Inhibition of 5α-RE: Pygeum africanum extract PY 102, and Urtica dioica extract UR 102, inhibited the 5α-RE activity in a concentration dependent manner. Whereas UR 102 extract was only able to influence the enzyme activity at high concentrations (~12 mg/ml) and its ED50 being calculated as 14.7 mg/ml, the PY 102 extract showed a much higher activity starting with low concentrations (0.1 mg/ml) its ED50 being calculated as 0.78 mg/ml. When compared with the effects of UR 102, the combination of both extracts, PHL-00801 (Prostatonin®), led to a similar inhibition of the enzyme (ED50 14.15 mg/ml).

Inhibition of AR: The PY 102 extract showed a concentration dependent and strong activity (ED50 = 0.98 mg/ml). The activity of the UR 102 extract was also concentration dependent (ED50 = 3.58 mg/ml).

The combination of both extracts, PHL-00801 (Prostatonin®) showed a synergistic action and significantly (p = 0.05) increased the AR-inhibitory activity in concentrations as low as 0.1 mg/ml (ED50 0.24 mg/ml).

These observations are an explanation for the beneficial effects of PHL-00801 (Prostatonin®) observed in the clinical studies on BPH.

Key words: 5α-reductase, aromatase, benign prostatic hyperplasia, Urtica dioica UR 102 extract, Pygeum africanum PY 102 extract, PHL-00801 (Prostatonin®).

Introduction

Benign prostatic hyperplasia (BPH) is an androgen-dependent disease which afflicts nearly 50% of males over the age of 60 and is seen in almost all males of age 80. The pathogenesis of benign prostatic hyperplasia is not yet fully understood.

The most attractive hypothesis in the so-called Dihydrotestosterone-Hypothesis. After that, dihydrotestosterone, the 5α-reduced metabolite of testosterone, is seen as a causative factor in the progression of prostatic hyperplasia. Furthermore, also the metabolite of dihydrotestosterone, androstanediol seems to contribute to the pathogenetic process. Support for that hypothesis comes from clinical studies of males who are genetically deficient in the dihydrotestosterone producing enzyme, the testosterone 5α-reductase. In these subjects no prostate growth appears (Geller, 1989; Metcalf et al., 1989; Henderson, 1991).

Further support comes from the data with the specific 5α-reductase inhibitor Finasteride showing a decrease in
dihydrotestosterone levels in prostatic tissue and concomitantly a decrease in prostatic growth (Stoner and Finasteride Study Group, 1992).

An increased ratio of plasma estrogen/testosterone in elderly men together with reports of the presence of estrogen receptors in the prostatic stroma, indicate that estrogen also may be involved in the development of BPH (Geller, 1989). In the male estrogens are probably formed by the conversion of androstenedione to estrone and estradiol-17β by the enzyme aromatase mainly located in adipose tissue (Griffiths et al., 1991). Furthermore, the presence of aromatase activity in BPH tissue has been demonstrated (Marzkin and Soloway, 1992). This raises the possibility of a regulation of intraprostatic levels of estrogen independent of circulating plasma levels (Stone et al., 1986; Ayub and Level, 1990). Estrogen can promote smooth muscle hyperplasia (Bruengger et al., 1986). In consequence the use of aromatase biosynthesis inhibitors e.g. aromatase inhibitors for the treatment of BPH has been suggested (Habenicht et al., 1986).

On the other hand there are authors who deny the existence of the estrogen forming enzyme in prostatic and BPH tissue (Perel and Killinger, 1983; Brodie et al., 1989; Rowlands et al., 1991).

PHL-00801 (Prostatonin®) is a 12:1 combination of the extract UR 102 of the plant Urtica dioica, and PY 102 of the plant Pygeum africanum, both of which are used for prostate complaints.

Animal experiments demonstrated that Pygeum extracts regenerate the prostate epithelium of senescent rats and dogs and that they activate prostate secretion. Moreover they inhibit the basic-fibroblast growth factor induced cellular proliferation (Paubert-Braquet et al., 1994). The effect of Urtica extracts based on the inhibition of the membrane Na+ K+-ATPase activity in the prostate, which may subsequently suppress prostate-cell metabolism and growth (Hiรฉnaro et al., 1994). Other studies suggest that Urtica extracts inhibit the aromatase enzymes (Kraus et al., 1991), reduce the binding activity of sex hormone-binding globuline (Gansser and Spiteller, 1995) and block the epidermal growth factor receptors (Wagner et al., 1995).

Clinical studies demonstrated that, by a two months treatment with PHL-00801 (Prostatonin®), urination in longer intervals and amounts is achieved (Mantanari et al., 1991; Camponovo and Maranta, 1990; Krzeski et al., 1993).

The mechanism of action of this combination – especially of Pygeum africanum – is not yet clarified.

It was therefore the aim of the present study to investigate whether or not PHL-00801 (Prostatonin®) or the extracts from Urtica dioica and Pygeum africanum are able to influence 5α-reductase and/or the aromatase enzyme and if the extracts could possibly exert synergistic activities.

Material

Test substances

The following test substances were used:

*Pygeum africanum* extract (PY 102) Batch 23442

The bark is extracted with methylene chloride, the ratio bark to extract is 200:1.

The extract is a native extract, which is standardized on a content of 13 (± 1.3)% sterols (determined as β-sitostanol).

*Urtica dioica* extract (UR 102) Batch 8821972

The roots are extracted with 30% methanol, the ratio roots to extract is 10:1.

The extract is a native extract.

Both samples were supplied by Pharmaton SA, CH-6934-Lugano-Bioggio.

As a positive control for 5α-reductase assays (N-[2-(3-Carboxy)propoxyphenyl]-β-methyl-4-n-pentyl-cinnamimide), a compound previously described in the European Patent Application, application number 88304127.9, was used (Nakai et al., 1988). This substance was synthesized in the laboratories of Dr. Karl Thomae GmbH, D-88397 Bieberach, according to the method described in the patent.

As a positive control for aromatase assays, aminogluthethimide was used. This substance was previously described in the European Patent number 290378 and synthetized in the laboratories of Prof. R. W. Hartmann, Université des Saarlandes, D-66041 Saarbrücken.

Reagents

Testosterone-[4-14C] (specific activity 57 mCi/mmol) was purchased from Amersham International PLC (code CFA 129). NADPH tetrasodium salt was supplied by Boehringer Mannheim, cat. No. 107816.

[1β, 2β-3H]testosterone (specific activity 48.3 Ci/mmol) was purchased from New England Nuclear (NEN), Dreieich [NET 187]. Glucose-6-phosphate-dehydrogenase (346 U/mg protein) [22822], dextran 60 (mw 60 000-90 000) [18692] and charcoal Novit A was purchased from Serva, Heidelberg [30890].

Animals

Male Wistar rats, strain Chhb:THOM (SPF), weighing 300–350 g were used throughout the experiments. Animals with free access to water were maintained on a standard laboratory chow diet (NAFAG 8577) and fed ad libitum.

They were sacrificed by cervical dislocation and the ventral lobes of prostatic glands were pooled after removal of surrounding connective tissues.
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Biological material

Freshly delivered human term placentas were obtained by St. Josef Hospital, Saarbrücken-Dudweiler.

Methods

HPLC – fingerprint analysis –

Separation parameters for the Pygeum africanum extract PY 102:

Column: Spheri 5 RP 8 μm (250 x 4.6 mm) Brownlee
Pre-column: Acquapore RP 300 7 μm (30 x 2.1 mm) Brownlee
Eluent for gradient: A, MeOH-H₂O (95:5, v/v); B, CH₃CN
Gradient program:

<table>
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<tr>
<th>t (min)</th>
<th>% A</th>
<th>% B</th>
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<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
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</tr>
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<td>9</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>15.5</td>
<td>55</td>
<td>45</td>
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Flow: 1.5 ml/min
Detection wavelength: 323 nm
Preparation of the sample: Dissolve 125 mg PY 102 in 50 ml CHCl₃.
Injection volume: 10 μl

Separation parameters for the Urtica dioica extract

UR 102:

Column: Nucleosil 100 C 18 7 μm (250 x 4 mm) Macherey-Nagel
Fluent: KH₂PO₄ 0.01 M – CH₃CN (80:20, v/v)
Flow: 1.0 ml/min
Detection wavelength: 343 nm
Preparation of the sample: Dissolve 150 mg UR 102 in 10 ml MeOH, filtrate, (Chromafil 0.22 μm) and evaporate. Dissolve the residue in 2 ml MeOH.
Injection volume: 20 μl

5α-reductase

The method used has been previously described by Shimazaki et al. (1971). After that, the prostatic tissues (2.5 g) were homogenized, filtered through nylon cloth (DIN 24-250) and then centrifuged. After resuspending and another centrifugation step the resulting precipitate was suspended in buffer and used as a source of enzyme.

The whole procedure described so far was performed at a temperature of < 4 °C.

Fig. 1. HPLC profile of Pygeum extract PY 102 (top), with the characteristic peak of n-docosyl ferulate at 9.45 min and HPLC profile of Urtica dioica extract UR 102 (bottom), with the characteristic peak of scopoletine at 9.01 min. Chromatographic conditions: see under methods.
A reaction mixture of [4-14C]-testosterone (1.5 nmol, 1.5 x 10^5 cpm), NADPH, enzyme source prepared together with several concentrations of the extracts were incubated for 60 min at 37 °C in a shaking water bath. Enzyme reaction was stopped by addition of a mixture of chloroform and methanol (1:2, v/v), and then centrifuged. An aliquot of the supernatant (50 μl) was spotted on silica gel thin layer plates (Merck Nr. 5721). The plates were developed with a mixture of chloroform and diethylether (9:1, v/v).

The zones corresponding to 5 α-dihydrotestosterone on each plate were scraped off and dissolved with methanol. Thereafter scintillation cocktail was added. Radioactivity was counted in a liquid scintillation spectrometer (Packard TRI-CARB 2000 CA). Proteins were determined according to the method described by Lowry et al. (1951).

All samples were run in quadruplicates with at least 8 control samples within each experiment.

A patent-known 5 α-reductase inhibitor served as a positive control. Within all experiments run the mean inhibitory effect of this compound in a concentration of 10^-6 Mol/l was 61.06% ± 2.51 (n=15).

Values given are means ± SE of at least 2 (UR 102) or 3 (PY 102; and PY 102 plus UR 102) independent experiments.

Ethanol was used to suspend the extracts, the final ethanol concentration of control and inhibitor incubation was 5%.

Resulting dpm values (after substraction of blind control values) were normalized to dpm dihydrotestosterone per mg soluble (prostatic) protein and 100 000 dpm testosterone.

Therefore original values were normalized by multiplication them with the corresponding factors.

**Aromatase**

The method of Thompson and Siiteri (1974) was used with modifications. The placenta was transported in KCl-solution (0.15 M; 4 °C) and immediately processed at a temperature of 4 °C. After washing with KCl-solution, the placenta was cut into pieces using a scalpel and washed again.

The pieces were then thoroughly minced using scissors and repeatedly washed. After this cold sucrose solution (0.25 M) was added (1 ml/g; tissue wet weight approximately 220 g) and the tissue homogenized using an ultraturrax (maximal speed: 24 000 rpm; 10 times for 20 s; intervals: 1 min each).

After filtration through a nylon cloth the homogenate was centrifuged at 20'000 g for 30 min. The pellet was discarded and the supernatant centrifuged at 150'000 g for 45 min. After discarding the supernatant the microsomal pellet was resuspended in 400 ml of phosphate buffer (0.05 M, pH = 7.4). After centrifugation (150'000 g, 45 min) the washing procedure was repeated. The pellet obtained was resuspended in approx. 16 ml of phosphate buffer and frozen in 1 ml portions at -30 °C (the enzyme activity remained constant for some months). The protein concentration was determined using the method of Lowry et al. (1951). The overall yield was 163 mg protein. Before performing the aromatase inhibitory test, the protein concentration of the enzyme suspension was adjusted to 5 mg protein/ml suspension.

The assay was performed according to the method of Graves and Salhanick (1979) with modifications (Hartmann and Batzl, 1986). In the assay the enzyme activity was monitored by measuring the 3H2O formed by [1β,2β-3H]testosterone during aromatization.

Each incubation tube contained 0.225 μCi (4.4 pmol) of [1β,2β-3H]testosterone, unlabelled testosterone (2.5 nmol), NADPH (1 μmol), glucose-6-phosphate (10 μmol), glucose-6-phosphatedehydrogenase (1.25 U) and plant extract (0-12 mg/ml) in phosphate buffer (0.05 M, pH = 7.4). Ethanol was used for dissolving the extracts. The final ethanol concentration of control and inhibitor incubations was 5%. Each tube was preincubated for 5 min at 30 °C in a shaking water bath. Microsomal protein (0.5 mg) was added to start the reaction. The total volume of each incubation was 0.5 mL. The reaction was terminated by withdrawing 100 μl aliquots at 0, 7, 14, and 21 min and pipetting them into 200 μl of a cold 1 mM HgCl2 solution. After addition of 200 μl of an aqueous dextran-coated charcoal (DCC) suspension (2%), the vials were shaken for 20 min and centrifuged at 1’500 g for 5 min to separate the charcoal-adsorbed steroids. Aliquots of the supernatant were assayed for 3H2O by counting in a scintillation mixture in a LKB liquid scintillation spectrometer (Racbeta).

Each control or inhibitor incubation was run in duplicate. The amount of product formed was averaged (after substraction of blind control values). At each concentration of inhibitor the samples were removed from each assay tube at three time points. The results were plotted on a graph of product against time of incubation. The resulting graph was used to determine the inhibition of enzyme reaction at the corresponding concentration of inhibitor by comparing the values to those of control samples (no inhibitor) that were run simultaneously. The inhibition values were used to determine the EDso-values. For their determination each inhibitor was tested in six (five) appropriate concentrations (PY 102: 3-0.01 mg/ml; UR 102: 12-0.036 ml/mg). The percent inhibition was plotted vs. the concentration of inhibitor. From this the concentration causing 50% inhibition (EDso-value) was calculated. Each experiment was run six times. Aminogluthethimide, the only non-steroidal aromatase inhibitor, which is commercially available served as a positive control. Within all experiments run the mean inhibitory effect of this compound was: EDso = 16 ± 1.7 μM (N = 18).
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Data analysis

The student t-test was used for statistical comparison of the data with the conventional p = 0.05 as the level of significance.

Results

Inhibition of 5 α-reductase

The extract DR 102 was tested in concentrations ranging from 36 mg/ml to 0.12 mg/ml. The extract DR 102 markedly reduced enzyme activity in concentrations ≥ 12 mg/ml. The ED$_{50}$ value was calculated as 14.7 mg/ml [Fig. 2] [Table 1].

Table 1.

<table>
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<th>ED$_{50}$ (mg/ml)</th>
<th>ED$_{50}$ (mg/ml)</th>
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<tr>
<td></td>
<td>Inhibition of 5 α-REDUCTASE</td>
<td>Inhibition of AROMATASE</td>
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<tr>
<td>UR 102</td>
<td>14.70</td>
<td>3.58</td>
</tr>
<tr>
<td>PY 102</td>
<td>0.78</td>
<td>0.98</td>
</tr>
<tr>
<td>Prostatonin®</td>
<td>14.15</td>
<td>0.24</td>
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ED$_{50}$ of Urtica dioica extract UR 102, Pygeum africanum extract PY 102 and their combination in a 12:1 ratio (Prostatonin®).

The extract PY 102 was tested in concentrations ranging from 10 mg/ml to 0.01 mg/ml. Pygeum africanum extract PY 102 inhibited the 5 α-reductase activity in a concentration-dependent manner. As low concentrations as 0.1 mg/ml led to a significant decrease in enzyme activity. Almost total blockade of the enzyme was achieved with a concentration of 10 mg/ml. The ED$_{50}$ value was calculated as 0.78 mg/ml [Fig. 3].

In the combination experiments (for PHL-00801 Prostatonin®) both extracts were dissolved in 5% ethanol. To mimic the relative abundances of Urtica dioica and Pygeum africanum extracts within the preparation Prostatonin® to each concentration of PY 102 the twelve fold amount of UR 102 was added.

The combination was tested in a concentration range of 3 to 0.01 mg/ml PY 102, and of 36 to 0.12 mg/ml UR 102.

Compared to the effects of PY 102 alone only in the concentration of 3 mg/ml PY 102, UR 102 (36 mg/ml) was able to further augment the inhibitory effect of PY 102.

When compared to the effects obtained with UR 102 alone, the combination of PY 102 plus UR 102 led to a similar inhibition of the enzyme, the ED$_{50}$ value (expressed on the basis of UR 102 concentrations) was calculated as 14.15 mg/ml [Fig. 4].

Inhibition of aromatase

The extract PY 102 was tested in concentrations ranging from 3 mg/ml to 0.01 mg/ml. A concentration dependent inhibition was observed. At a concentration of 3 mg/ml the extract showed a strong inhibition of human placental aromatase (approximately 80%). The ED$_{50}$ value was calculated as 0.98 mg/ml [Fig. 5].

The UR 102 extract was tested in concentrations from 12 mg/ml to 0.036 mg/ml. In this case, too, a concentration dependent inhibition was observed. At the maximum concentration tested (12 mg/ml) a strong inhibition was shown (78–95%). The ED$_{50}$ value was calculated as 3.58 mg/ml [Fig. 6].

The combination experiments were performed to mimic the relative abundances of Urtica dioica and Pygeum africanum extracts in the preparation PHL-00801 (Prostatonin®).
The enzyme 5α-reductase catalyzes the reaction from testosterone to dihydrotestosterone. Dihydrotestosterone plays a major role for the growth of the prostate gland. Further research is needed to fully understand the potential of Pygeum africanum and Urtica dioica extracts in inhibiting these enzymes and their combined effects on prostate health.
Inhibition of 5 α-reductase and aromatase by PHL-00801 (Prostatonin®) observed in the clinical studies on benign prostatic hyperplasia. These in vitro results have to be evaluated in further vivo experiments.

References


Nakai, H., Terashima, H. and Arai, Y.: European Patent Application, application number 88304127.9, date of filing 06.05.1988.

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