**Serenoa repens (Permixon®): A 5α-Reductase Types I and II Inhibitor—New Evidence in a Coculture Model of BPH**

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**BACKGROUND.** The aim of this study was to determine the effect of the phytotherapeutic agent, Permixon®, on a novel coculture model of benign prostatic hyperplasia (BPH) in an effort to better understand the mode of action of the drug in vivo.

**METHODS.** The effect of Permixon®, at the calculated therapeutic concentration, on the activity of 5α-reductase isoenzymes was evaluated utilizing a pH-specific assay. Prostate-specific antigen (PSA) secretions into the medium were measured in the presence and absence of Permixon® and quantified by an ELISA assay. The morphological patterns before and following Permixon® treatment were also examined by electron microscopy. All results were compared to controls.

**RESULTS.** Permixon® at a concentration of 10 μg/ml (calculated plasma concentration in patient receiving recommended therapeutic dosage) was shown to be an effective inhibitor of both 5α-reductase types I and II isoenzymes without influencing the secretion of PSA by the epithelial cells, even after stimulation with testosterone. The morphology of Permixon®-treated cells was found to be markedly different from that of untreated controls. Cells which had been treated with the drug demonstrated extensive accumulation of lipids in the cytoplasm and widespread damage of intracellular membranes, including mitochondrial and nuclear membranes.

**CONCLUSIONS.** Permixon® is an effective dual inhibitor of 5α-reductase isoenzyme activities in the prostate. Unlike other 5α-reductase inhibitors, Permixon® induces this effect without interfering with the cells’ capacity to secrete PSA, thus permitting the continued use of PSA measurements for prostate cancer screening. *Prostate* 40:232–241, 1999.

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**KEY WORDS:** coculture; Permixon®; 5α-reductase type I; 5α-reductase type II; BPH; PSA

**INTRODUCTION**

Endocrine therapy for benign prostatic hyperplasia (BPH) has been employed for nearly 100 years, but the drawback of most methods of androgen deprivation, whether luteinizing hormone-releasing hormone (LHRH) analogues, progesterones, or antiandrogens, is that they all induce, to varying degrees, hypogonadism and impotence [1–3]. As a result of these side effects, interest in a new class of drugs including 5α-reductase (5αR) inhibitors and phytotherapeutic agents has emerged. Working on the premise that dihydrotestosterone (DHT) is a critical factor in the growth of the prostate [4–7] and the development of BPH, several drugs have been developed to reduce the activity of androgens on target cells. This has been achieved by inhibiting the action of the 5α-reductase isoenzymes, which are responsible for the metabolism of testosterone to DHT and are known to be expressed...
in the normal and pathological human prostate [8]. Several of these 5α-reductase inhibitors are now available [9–12], while others are being developed by various companies. These drugs are largely of two types: specific, synthetic inhibitors such as finasteride, and phytotherapeutic agents such as Permixon® (Pierre Fabre Medicament, Castres, France).

Plant extracts have been used in many phytotherapeutic medicines for the treatment of BPH for many years [13,14]. Due to their mixed composition, they exert several different types of activity and have not been studied in as great detail as their synthetic counterparts.

The synthetic drug currently available, finasteride, only inhibits the action of one isoenzyme (type II), while having little or no effect on the other isoenzyme at the recommended therapeutic dosage [9]. Permixon®, a hexane lipid/sterol extract of the fruits of the dwarf palm Serenoa repens, has been shown to act as a non-competitive inhibitor for both isoforms of 5α-reductase (type I and type II) in a baculovirus-directed insect cell expression system [12], with Ki values of 7.2 mg/l and 4.9 mg/l, respectively. The drug also induces a significant reduction in DHT and epidermal growth factor (EGF) concentrations in human BPH following 3 months of treatments [15]. In addition, Permixon® has been shown to have several other effects and actions [16–18] in different experimental systems, and these may have an influence on the growth of the prostate. Among the effects reported are inhibitory effects on growth factor-induced proliferation of human prostate cell cultures [17], and inhibition of prolactin receptor signal transduction in Chinese hamster ovary (CHO) cells [19]. The latter effect resulted in reduced K+ channel conductance and a reduction in the activity of protein kinase C (PKC).

Earlier experimental studies employed either transfected cells, prostate cancer cells, or foreskin fibroblasts where only one of the isoenzymes was expressed [12,16,17,19]. Furthermore, there were some conflicting results with regard to the inhibitory activity of Permixon® on 5α-reductase [12,20,21]. We have also noted that none of the earlier studies so far reported had examined the effect of Permixon® on prostate-specific antigen (PSA) expression. Since some of the 5α-reductase inhibitors appear to inhibit the secretion of PSA in prostatic cells, we wondered whether Permixon® might exert a similar effect.

Therefore, this report was aimed at answering some of the questions regarding the impact of Permixon® on 5α-reductase isoforms and PSA in vitro and in vivo. To demonstrate this, we employed a novel in vitro model for BPH which expresses many of the phenotypic characteristics of the prostate, including both isoforms of 5α-reductase, PSA, and androgen receptors [22], thus mirroring more closely the conditions seen in an in vivo system.

**MATERIALS AND METHODS**

**Preparation of Permixon®**

Permixon® was obtained as a hexane extract from Pierre Fabre Medicament (Castres, France). The hexane was evaporated to leave the solid extract. The solid was dissolved in 10 ml of ethanol, to give a concentration of 10 mg/ml. This stock solution was further diluted in appropriate media to provide a working solution of 1 mg/ml. All controls were supplemented with an equivalent volume of ethanol.

**Establishment of Coculture Cell Model**

BPH tissue was obtained from men undergoing transurethral resection of the prostate. The histological status of the tissue was checked by an independent pathologist. Primary cultures of fibroblast and epithelial cells were established as previously described [23,24]. The identity and purity of the separate cultures were confirmed by means of both immunohistochemistry and phase-contrast microscopy. From the primary cultured cells, cocultures of prostate epithelial and fibroblast cells were prepared as described previously [22], by fitting the cell culture plates with a microporous membrane to separate the two populations while maintaining communication by means of diffusible factors. Cocultures were maintained in WAJC 404:RPMI-1640 + 10% fetal calf serum (FCS) (1:1 v/v) media at 37°C in 5% CO₂.

**Cell Proliferation**

**Primary cultured cells.** Epithelial cells were plated at a density of 5,000 cells/well in 100 μl WAJC 404 supplemented with insulin, and fibroblast cells were plated at a density of 10,000 cells/well in 100 μl RPMI-1640 supplemented with 10% FCS. The proliferation of the cells was compared to controls, which received no Permixon® but did receive a concentration of alcohol comparable to that in the Permixon®-treated samples. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25], which was standardized against manual cells counts. All results are expressed as the mean ± SEM of three replicates.

**Cocultured cells.** Cocultured epithelial (50,000) and fibroblast (5,000) cells were treated with Permixon® at varying concentrations (0–100 μg/ml) for a period of 5 days, and the proliferation of epithelial and fibroblast
cells was compared to controls which received no Permixon®. Cell proliferation was determined using the MTT assay, which was standardized against cell counts. All results are reported as the mean ± SEM of three replicates.

Measurement of Functional 5α-Reductase Activity

Cocultured epithelial and fibroblast cells grown in the presence and absence of Permixon® (0—100 μg/ml) for a period of 4 days were assayed for 5α-reductase activity, as described previously [26,27]. Briefly, cells were harvested by trypsinization, pelleted by centrifugation, resuspended in RPMI-1640 supplemented with 10% FCS, and counted. Cells were resuspended in 600 μl sodium phosphate buffer (4 mmol/l) either at pH 7.5 (5α-RI) or pH 5.5 (5α-RII); the buffer contained sucrose (0.32 mol/l) and dithiothreitol (1 mmol/l). Cell suspensions were added to glass tubes containing substrate [3H] testosterone ((1,2,6,7-3H) testosterone, specific activity 105 Ci/mmol; Amersham International, Little Chalfont, Buckinghamshire, UK) (20 nmol/l; 1 μCi), a NADPH regenerating system, and 10 μg/ml Permixon®. Samples were adjusted to 1 ml by the addition of sodium phosphate buffer at the appropriate pH. Tubes were then incubated at 37°C for 30 min in a shaking water bath. The reaction was stopped by the addition of an equal volume of diethylether containing 500 cpm [14C; 50 mCi/mm] DHT (Amersham International) and 25 μg each of unlabeled 3α-androstanediol, 3β-androstanediol, testosterone, DHT, and androstenedione. Steroids were extracted by evaporating to dryness in a vacuum oven at 40°C, and the residue was resuspended in ethanol and applied to glass silica gel TLC plates (Gelman Sciences, Ann Arbor, MI). Separation was carried out by TLC using dichloromethane-diethyl ether (9:1 vol/vol). The metabolites were visualized by spraying with phosphomolybdic acid reagent spray. The radioactive metabolites were quantified using a Tri-carb liquid scintillation counter (Canberra Packard, Pangbourne, Berkshire, UK), and 5α-reductase type I and type II activities were assessed by measuring the conversion of testosterone to DHT, as previously described [26,27].

PSA Measurement and Expression

Immunoenzymetric assay. Conditioned media from six wells (10,000 epithelial cells/well) of Permixon®-treated, cocultured epithelial cells were collected after 4 days of incubation and dialyzed for 24 hr against dH2O in membranes with a 6-8,000 D molecular weight cutoff (Membrane Filtration Products, Inc., San Antonio, TX). The dialysate was then lyophilized in a Lyoprep freeze drier and reconstituted in 100 μl of sterile dH2O, and PSA was measured using a Tandem- MP assay kit (Hybritech Incorporated, Liege, Belgium) according to the manufacturer’s protocol. Results were expressed as mean (ng/ml) ± SEM of three replicates and compared to data obtained for cocultured epithelial cells grown in the absence of Permixon®.

Immunohistochemistry of PSA. Epithelial cells were grown under coculture conditions in the presence and absence of Permixon® for 4 days before being fixed directly in six-well plates in 1% formaldehyde in phosphate-buffered saline (PBS; 0.27 M KCl, 0.137 M NaCl). After washing twice in Tris-buffered saline (TBS; 60 mM Tris, 137 mM NaCl), endogenous peroxidase activity was blocked with 3% H2O2 solution. Nonspecific binding of antibodies was blocked with 20% sheep serum for 20 min at room temperature. Incubation was carried out overnight at 4°C with antihuman PSA monoclonal antibody (Bionostics, Ltd., Wyboston, Bedfordshire, UK) diluted in TBS (1:50,000). The cells were then washed twice with TBS for 5 min and incubated in biotinylated sheep anti-mouse antibody (Dako, Ltd., High Wycombe, Buckinghamshire, UK) for 30 min at room temperature, followed by two washes in TBS. Horseradish peroxidase (HRP)-conjugated streptavidin was added. After 30 min of incubation, the cells were washed twice with TBS and developed with diaminobenzidine. Cells were counterstained with hematoxylin for 1 min. For each staining experiment, a negative control and a positive control (LNCaP cell line) were included.

Electron Microscopy Studies

Cocultured epithelial and fibroblast cells which had been incubated for 2 days in the presence and absence of Permixon® were fixed overnight and prepared for analysis by electron microscopy, as described previously [28]. Fixed samples were impregnated with EMIX resin and cut into 90-nm sections, which were mounted on 300-mesh copper grids before staining using the uranyl acetate/lead citrate method, as detailed previously [28]. After processing, the samples were visualized using a Jeol 100CXII transmission electron microscope (JEOL, Welwyn Garden City, Hertfordshire, UK) operating at 60 kV.

Statistical Analysis

Statistical significance was determined using a two-tailed Student’s t-test for comparison of two means, and all results are expressed as mean ± SEM.
RESULTS

Proliferative Response of Cells to Permixon®

Primary cultured epithelial and fibroblast cells. Primary cultured epithelial (Fig. 1) and fibroblast (Fig. 2) cells supplemented with increasing concentrations of Permixon® demonstrated a cytotoxic response to 100, 50, and 25 µg/ml concentrations of Permixon® when compared to controls (no Permixon®). Permixon® at 10 µg/ml had no effect on growth. Growth rate was assessed spectrophotometrically (540 nm) by MTT assay. Data are expressed as mean ± SEM of three replicates.

Cocultured epithelial and fibroblast cells. Unlike previous experiments on primary cultured cells, cocultured epithelial (Fig. 3) and fibroblast (Fig. 4) cells treated with increasing concentrations of Permixon® up to 100 µg/ml demonstrated no difference in proliferation patterns (Figs. 3, 4) when compared to controls. This indicates that at those concentrations, Permixon® exhibited no cytotoxic effects on the cocultured cells. Taking into consideration the recommended therapeutic dosage of 320 mg/day and the cytotoxic effect observed in primary cultured cells, it was decided to perform all subsequent experiments with a concentration of 10 µg/ml of Permixon® and confine these experiments to the coculture model, which has been shown to be a better representation of the in vivo prostate [22]. This dosage is comparable to the predicted physiological concentration of Permixon® administered to patients [29], assuming distribution in total body fluid.

Measurement of 5α-Reductase Types I and II Activity in Permixon-Treated Cocultured Cells

We compared the functional activities of the two 5α-reductase isoenzymes in Permixon®-treated (5 days) and untreated cocultures of epithelial and fibroblast cells. Cell suspensions (~100,000 cells/ml) obtained from cocultured epithelial (Fig. 5a) and fibroblast (Fig. 5b) cells exhibited high levels of 5α-reductase type I and type II activities, as measured by the conversion of testosterone to DHT at pH 7.5 (5α-R1) and pH 5.5 (5α-RII). The activity in epithelial cells was greater than the activity in fibroblast cells for both isoenzymes. However, in the presence of Permixon® (10 µg/ml), the cocultured cells showed a marked inhibition of both isoenzymes (P < 0.05) (Fig. 5a,b). The degree of inhibition when compared to controls for both isoenzymes was approximately similar in the cocultured epithelial cells (type I, 55.9%; type II, 60.7%).
more susceptible to Permixon® and demonstrated a greater degree of inhibition (90%) than the type I isoenzyme (22.9%).

Influence of Permixon® on PSA Expression in Cocultured Epithelial Cells

Conditioned media obtained from cocultured epithelial cells treated for 4 days with 10 µg/ml of Permixon® yielded significant concentrations of PSA (0.22 ± 0.0015 ng/ml) (Fig. 6), and these were of a similar order of magnitude as the concentrations produced by the cocultured epithelial cells (0.28 ± 0.002 ng/ml) (Fig. 6) in the absence of Permixon®. Furthermore, we noted that the significant increase (P < 0.05) in PSA secretion (0.4115 ± 0.003 ng/ml) following stimulation of the cells with exogenous testosterone (10 nM) was not in any way affected by the presence of Permixon® (10 µg/ml) (Fig. 6). Indeed, conditioned media from cells pulsed with testosterone in the presence of Permixon® yielded a mean PSA concentration of 0.43 ± 0.003 ng/ml, which was similar to the concentrations obtained in the absence of the drug. In all experiments, PSA measurements were normalized to cell counts.

We also undertook some PSA immunostaining experiments, employing a specific monoclonal antibody to the antigen. These demonstrated that the cocultured epithelial cells stained positively for PSA as did the Permixon®-treated epithelial cells, with the bulk of the staining being detected in the cytoplasm of the epithelial cells. Negative staining was also obtained for fibroblast cells derived from both Permixon®-treated and untreated cultures (results not shown).

Fig. 4. Growth response of cocultured prostate fibroblast cells to Permixon® at increasing concentrations (10, 25, 50, and 100 µg/ml) over a period of 5 days. Prostate fibroblast cells grown in coculture with prostate epithelial cells showed no growth effects when incubated with the drug at any of the concentrations employed in relation to controls (no Permixon®) except at the highest concentration of 100 µg/ml, which produced a statistically significant decrease in growth of the cells (*P < 0.05) following 4 days of treatment. Growth rate was assessed spectrophotometrically (540 nm) by MTT assay. Data are expressed as mean ± SEM of three replicates.

Fig. 5. 5α-reductase types I and II activity in cocultured epithelial (a) and fibroblast (b) cells. Cocultured cells were analyzed for 5α-reductase activity by employing a pH-specific assay to identify the two isoenzymes (type I, pH 7.5; type II, pH 5.5). The effect of 5 days’ treatment with Permixon® (10 µg/ml) on 5α-reductase activity was determined utilizing the same procedure. Each data set is the result of three separate experiments. Results are expressed as mean ± SEM. *P < 0.05.

Effect of Permixon® Treatment on Morphology of Prostate Epithelial and Stromal Cells Grown in Coculture

Cells grown in coculture in the presence of Permixon® (10 µg/ml) for 2 days were examined by electron microscopy for specific intracellular structures. The morphology of these cells was also compared to that of cocultured cells not treated with Permixon®. Cocultured epithelial cells (Fig. 7a) which had not been treated with Permixon® exhibited an abundance of tonofibrils, maintained a complex microvilli structure, and possessed a large number of secretory vesicles. Untreated cocultured fibroblasts (Fig. 8a), on the other hand, were characterized by the presence of
cilia, which are known to be specific to this type of cell and the formation of collagen. We also noted a large amount of Golgi apparatuses, confirming once again and the formation of collagen. We also noted a large cilia, which are known to be specific to this type of cell and the formation of collagen. We also noted a large amount of Golgi apparatuses, confirming once again and the formation of collagen. We also noted a large

cultured epithelial cells grown in the absence and presence of Permixon® (10 µg/ml). PSA was measured using a Tandem-MP ELISA kit. Each data set is the result of three separate experiments. Results are expressed as mean ± SEM. *P < 0.05.

Permixon®-treated epithelial (Fig. 7b) and fibroblast (Fig. 8b) cells showed marked structural differences when compared to untreated controls. Both cell types demonstrated accumulation of lipid droplets in the cytoplasm and wide-ranging damage to intracellular membranes, including nuclear and mitochondrial membranes. The effects of Permixon® on cell membranes are more obvious at higher magnification, when it becomes possible to visualize fragments of membrane disintegrating, including the nuclear membrane. These effects seem to be confined to intracellular structures and do not affect the cell membrane itself. Moreover, Permixon®-treated cells demonstrated polarization of the nucleus and condensation of the chromatin, which were not seen in untreated controls.

**DISCUSSION**

The hexane lipid-sterol extract of the dwarf palm (Serenoa repens) is used in a number of countries as a first-line treatment for patients with symptoms of outflow tract obstruction due to BPH [30–33]. The actual mechanism of action of Permixon® is not well-understood, but its effects are believed to be due to its ability to inhibit the action of both forms of 5α-reductase [34] in addition to inhibiting the binding of androgens to their receptors [20,35]. However, the evidence to support these observations has been gained from studying, in some reports, systems that are not directly comparable to human BPH, in which many of the phenotypic characteristics of the human gland are absent. Furthermore, in most of the earlier experiments, a cancer model was employed where one of the 5α-reductase isoenzymes was not expressed, and with the exception of LNCaP cell lines, none responded to androgens or secreted PSA [36–40].

We have developed a new human BPH model, which reflects several of the characteristics of the physiological system in man, including secretion of PSA, expression of functional androgen receptors, and the manifestation of both 5α-reductase isoenzymes [22]. The present study was undertaken mainly to investigate the effect of Permixon® on this new model as a way of understanding better the mechanism of action of the drug.

The work detailed in this report demonstrates that Permixon® is an effective inhibitor of both forms of 5α-reductase expressed in the prostate. In a similar manner, the data reported in this paper demonstrated that Permixon® does not interfere with the expression and production of PSA, even after androgen stimulation. The outcome of these experiments is in agreement with clinical trial data, which demonstrated no significant effect on PSA levels in BPH patients receiving the drug [29]. However, these results are in conflict with earlier in vitro data published by Sultan et al. [20], in which supraphysiological concentrations of Permixon® were used. In those studies, inhibition of the androgen receptors was reported, an observation subsequently confirmed by Ravenna et al. [18], who showed that at Permixon® concentrations higher than those employed in the present study, it was possible to induce an antiandrogenic effect. The concentrations of Permixon® used in those earlier studies were significantly higher than those predicted to be achievable in vivo.

The absence of any effect by Permixon® on measured levels of PSA would suggest that although Permixon® is inhibiting 5α-reductase, it is having little or no effect on other androgen-dependent processes which rely on the binding of androgens to their receptor. This is in contrast to other 5α-reductase inhibitors, such as finasteride, which in addition to their enzyme-inhibitory activities appear to alter the level of PSA expression by inhibiting the complex formed between androgen receptors and the steroid receptor binding consensus in the promoter region of the PSA gene [41]. It is evident from the present study that Permixon® does not interfere with the latter process, thus accounting for the absence of any effects on PSA secretion while maintaining potent 5α-reductase inhibitory activity.

The concentration of Permixon® used in our studies (10 µg/ml) is a predicted physiological concentration, assuming distribution in total body fluid achievable using the recommended therapeutic dosage. In these
Fig. 7. 

a: Electron micrograph of untreated cocultured epithelial cells. 

A (x3,888) demonstrates the large, round nucleus (N), cytoplasm (C), microvilli (M), and Golgi apparatus (G) which are indicative of epithelial cells. 

B (x15,984) demonstrates microvilli (M) at higher magnification and also shows tonofibrils (T) and mitochondria (Mi).

b: Electron micrographs of Permixon®-treated (10 µg/ml) cocultured prostate epithelial cells. 

A (x5,292) shows vacuolation (V) of the cytoplasm (C) and condensation of the chromatin (N). 

B (x56,160) demonstrates damage to the nuclear membrane, condensation of the chromatin (C) in the nucleus (N), tonofibrils, and damage to mitochondria (Mi). 

C (x138,240) demonstrates the typical effect of Permixon® on mitochondrial membranes (Mi).
experiments, we showed that both 5α-reductase isoenzymes can be inhibited effectively, while minimizing the cytotoxic effect of the drug in the cells. However, close inspection of the membranes of cells treated with Permixon®, in comparison to untreated cells, revealed that Permixon® disrupted the intracellular membranes of prostatic epithelial and fibroblast cells, including the nuclear membrane. 5α-reductase in the human prostate is dependent on being membrane-bound for its physiological activity [42] and is associated with the nuclear membrane [43]. The data reported herein represent strong evidence for the inhibition of 5α-reductase by Permixon® being a result of disruption of the enzymes’ microenvironment, thereby leading to inactivation of the 5α-reductase isoenzymes. While similar observations on the ultrastructure of prostate cells have been made by Ravenna et al. [18], who reported that intracellular damage was observed in LNCaP cells following incubation with Permixon®, those studies were confined to cells expressing the type I isoenzyme, since no other model was then available in which both isoforms of the enzyme were expressed. Therefore, the results reported in this paper suggest a novel method of enzyme inhibition by a drug without in any way disrupting the mechanism enhancing the androgen-responsive genes, thus accounting for the inability of Permixon® to interfere with the expression of PSA even after androgen stimulation. The hypothesis for the disruption of the membrane, leading to inactivation of the 5α-reductase isoenzymes, might also account for the absence of any inhibition in the activity of androgen receptor binding at the dosages employed. It is also interesting to note that in an earlier study on human BPH by Di Silverio et al. [44], androgen and estrogen receptors became localized in the cytoplasm of the prostate cells following treatment with Permixon®. The localization of the receptors may also be due to the receptors leaking out of the nucleus into the cytoplasm, a process facilitated by the Permixon®-damaged nuclear membrane.

Fig. 8. a: Electron micrographs of untreated cocultured fibroblast cells. A (×3,888) shows characteristic elongated nuclei (N), high levels of Golgi apparatuses (G), and cilia (Ci). B (×86,400) demonstrates a collagen fibril (F) produced by the cocultured fibroblast cells. b: Electron micrographs of Permixon®-treated (10 µg/ml) cocultured fibroblast cells. A (×15,984) shows general disruption of the cytoplasm of the cell and accumulation of lipids (L) in the cell. B shows damage to the Golgi apparatus (G) in a fibroblast cell treated with Permixon®.
So far, we have not determined whether Permixon® has any effect on the apoptotic profile of the human prostate. Several of the morphological changes observed in our coculture experiments, including the polarization of the nucleus and condensation of chromatin following treatment with Permixon®, suggest that Permixon® may be inducing apoptosis in both the epithelial and stromal cells of the prostate. Clearly this is an area worth further investigation, and is at present occupying some of our efforts as a way of further elucidating the mechanisms of action of Permixon® in the human prostate gland.

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REFERENCES


