THE SELECTIVITY AND SPECIFICITY OF THE ACTIONS OF THE LIPIDO-STEROLIC EXTRACT OF SERENOA REPENS (PERMIXON®) ON THE PROSTATE

COLIN W. BAYNE, MARGARET ROSS, FRANK DONNELLY AND FOUAD K. HABIB*

From the Prostate Research Group, University Department of Oncology, and the University Department of Pathology, Western General Hospital, Edinburgh, Scotland, United Kingdom

ABSTRACT

Purpose: To investigate the effects of the phytotherapeutic agent, Permixon®, on primary cultures of fibroblast and epithelial cells from the prostate, epididymis, testes, kidney, skin and breast and to determine the selectivity and specificity of the action of the drug.

Materials and Methods: All primary cultures were examined by electron microscopy before and following treatment with Permixon® (10 μg/ml.). In addition the apoptotic index was assessed by flow cytometry employing propidium iodide as a fluorophore. The impact of the drug on 5α-reductase (5αR) isoenzymes was also tested utilizing a pH specific assay.

Results: There were changes in the morphology of prostate cells after treatment including accumulation of lipid in the cytoplasm and damage to the nuclear and mitochondrial membranes; no similar changes were observed in other cells. Permixon® increased the apoptotic index for prostate epithelial cells by 35% and 12% in the prostate stromal/fibroblast. A lesser apoptotic effect was demonstrated in skin fibroblast (3%) whereas none of the other primary cultures showed any increase in apoptosis when compared with the controls. Permixon® was also an effective inhibitor of both 5αR type I and II isoenzymes in prostate cells, but other cells showed no inhibition of 5αR activity following treatment with the plant extract.

Conclusions: This investigation demonstrated the selectivity of the action of Permixon® for prostate cells. The morphological changes in the prostate are accompanied by an increase in the apoptotic index along with an inhibition in the activity of the nuclear membrane bound 5αR isoenzymes. No similar changes were observed in any of the other cells under investigation.

KEY WORDS: BPH, 5α-reductase, cell morphology, apoptosis, Permixon®

The treatment of benign prostatic hyperplasia (BPH) has until now relied mostly on surgery. However with an increasing incidence of the disease, there is additional pressure to develop more effective chemical alternatives to treat this condition thereby providing a reasonable option to surgical techniques.

There are at present a number of medical therapies available to relieve symptoms of BPH including 5α-reductase inhibitors (5αR), alpha adrenergic receptor blockers and plant extracts (phytotherapeutic agents). While phytotherapeutic agents have been used for many years,¹ most have not been studied in great detail and there is a dearth of information on their mode of action. Their mixed composition lends to their capacity to exhibit several pharmacodynamic properties accounting for their multiple mechanism(s) of action though these remain to be elucidated.

Permixon®, a n-hexane lipido/sterolic extract of the fruits of the American dwarf palm (saw palmetto) tree Serenoa repens, also known by its botanical name Sabal serrulata, is arguably the most popular and most studied phytotherapeutic BPH drug.² Randomized, double blind studies have shown Permixon® to be as effective as the uro-selective alpha-adrenergic blocker, alfuzosin and the 5αR inhibitor, finasteride, in achieving subjective and objective improvement.³, ⁴ Furthermore meta analysis of all available clinical trial data of the Permixon® preparation revealed a significant improvement in peak flow rate and reduction in nocturia greater than with placebo.⁵, ⁶ This agent demonstrates a number of characteristics, including the ability to inhibit the activity of both isoforms of 5αR in vitro⁷, ⁸ to decrease human prostate dihydrotestosterone concentrations in vivo⁹ and to block the receptor binding androgens in target cells.¹⁰ There is also evidence that Permixon® may act as an anti-inflammatory agent inhibiting both phospholipid A2 and the oxidative enzymes responsible for the synthesis of the pro-inflammatory eicosanoids.¹¹

Although Permixon® is now used as first line drug in the medical treatment of BPH in many countries due to its beneficiary effects on BPH symptoms and urinary flow rate¹²–¹⁴ there are no data demonstrating the selectivity and specificity of the drug toward the human prostate. Therefore this study was undertaken to determine the activity of Permixon in a variety of cells derived from the prostate and several other human organs. The parameters chosen for analysis were based on recent observations⁸ underlying a possible mechanism of action for the drug in target cells.

MATERIALS AND METHODS

Tissue culture media and reagents. Unless otherwise stated, all media and reagents were acquired from Life Technologies, Gibco Brl, Paisley, Renfrewshire, UK.

Chemicals. All chemicals were acquired from Sigma, Poole, Dorset, UK unless otherwise stated. Permixon®, Permixon® was obtained as the n-hexane extract of Serenoa repens from Pierre Fabre Medicament (Castres, France). The n-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

* Requests for reprints: Prostate Research Group, University Department of Oncology, Western General Hospital, Edinburgh EH4 2XU, Scotland, UK. Supported by Pierre Fabre Medicament.
diluted in appropriate media to provide a working solution of 1 mg./ml. Permixon® was employed at a concentration of 10 μg/ml. in all experiments; this concentration was based on plasma levels in patients receiving the recommended dosage. All controls were supplemented with an equivalent volume of ethanol.

Establishment of primary cell cultures of prostate and other tissues. Prostate stromal and epithelial cells were obtained by the collagenase digestion of resected BPH tissue samples as previously described.15, 16 Stromal/fibroblast cells were maintained in RPMI 1640 supplemented with 10% FCS whereas epithelial cells were grown in WAJC 404 medium supplemented with 0.5% FCS both at 37°C in 5% CO₂.

All other primary cultures were prepared employing variations of the respective methods: epididymal cells;17 testicular cells;18 kidney cells;19 skin fibroblasts;20 and breast fibroblasts.21 All protocols employed digestion of the tissue with collagenase. The cells were then maintained in RPMI1640 supplemented with 10% FCS in 5% CO₂ at 37°C. All epithelial cells entered into this investigation were from passage 1 and all stroma/fibroblast were below passage 3. In addition to the morphological examinations by electron microscopy, the identity and purity of all the primary cultures entered in this study were confirmed by phase contrast microscopy and immunohistochemistry as detailed previously.17–21

Electron microscopy: Cells which had been incubated for 24 hours in the presence and absence of Permixon® (10 μg/ml.) were fixed overnight and prepared for analysis by electron microscopy as described previously.22 Fixed samples were impregnated with EMIX resin and cut into 90 nm. sections and impregnated with EMIX resin and cut into 90 nm. sections before staining. All protocols employed digestion of the tissue with collagenase. The cells were then maintained in RPMI1640 supplemented with 10% FCS in 5% CO₂ at 37°C. All epithelial cells entered into this investigation were from passage 1 and all stroma/fibroblast were below passage 3. In addition to the morphological examinations by electron microscopy, the identity and purity of all the primary cultures entered in this study were confirmed by phase contrast microscopy and immunohistochemistry as detailed previously.17–21

Flow cytometric analysis of apoptosis. To determine whether the morphological changes seen in some of our primary cell cultures were characteristic of apoptosis, flow cytometric analysis was carried out on cell suspensions (10,000 cells/well) for each of the primary cultures investigated. Further suspensions were stained with 1.5 ml. of a solution containing 25 μg/ml. propidium iodide, 0.1 mm. EDTA, 0.01 mg./ml. DNase-free RNAses and 0.3% saponin in PBS pH 7.4. The samples were incubated in the solution for 5 hours at 4°C prior to cell cycle analysis on a FACS machine (Becton Dickinson, California, USA) interfaced with an IBM computer. A single argon laser beam was used to excite the propidium iodide dye. Each analysis was carried out on 10,000 cells at a rate of about 500 cells/second. Statistics were performed using MODFIT 5.2 software.

5α-reductase isoenzyme activities. 5α-R activity was assayed in the presence and absence of Permixon® as described previously.8, 24 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 (5αR type I) or pH 5.5 (5αR type II) which contained sucrose (0.32mol/l.) and dithiothreitol (1 mmol/l.). Cell suspensions were added to glass tubes containing substrate [3H] testosterone [(1,2,6,7-TH) testosterone, specific activity: 105 Ci./mmol.; Amersham International, Bucks, UK] (20 nmol/l.; 1 μCi), a NADPH regenerating system and 10 μg./ml. Permixon®. Samples were added to 1 ml. by the addition of sodium phosphate buffer at the appropriate pH. Tubes were then incubated at 37°C in 5 minutes in a shaking water bath. The reaction was stopped by the addition of an equal volume of diethylether containing 500 cpm [14C, 50 mCi/ mmol.] DHT (Amersham) and 25 μg. each of unlabelled 3α- androstenediol, 3α-androstenedioli, testosterone, DHT and androstenedione. Steroids were extracted by evaporating to dryness in a vacuum oven at 40°C and the residue resuspended in ethanol and applied to glass silica gel TLC plates (Gelman Sciences, Michigan, USA). 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) and 5αR type I (5αR-I) and type II (5αR-II) activities in the presence and absence of Permixon® were assessed by measuring the conversion of testosterone to DHT as previously detailed.8, 24

Statistical analysis. All results were expressed as mean ± S.D. and statistical significance was determined using the two-tailed Student’s t test.

RESULTS
The impact of Permixon® on the morphology of the primary cell cultures. Primary cell cultures were grown in the presence of Permixon® (10 μg/ml.) for two days then examined by electron microscopy for specific intracellular structures. The morphology of these cells was also compared with that of primary cultures not treated with Permixon® (figs. 1 and 2). All epithelial cells grew in monolayer and exhibited a microvilli structure whereas primary fibroblast were characterized by the presence of cilia which are known to be specific to this type of cell and large amounts of Golgi (figs. 1 and 2). On treatment with Permixon® prostate fibroblast and epithelial cells showed marked structural differences when compared with untreated controls (fig. 1, A-D). Both cell types demonstrated acceleration of lipid droplets within the cytoplasm, damage to the nuclear membrane and disruption of the organelles (fig. 1, A-D). Moreover in some prostate cells we noted the polarization of the nucleus and condensation of the chromatin (figs. 1, B, D).

In contrast the treatment of non-prostate derived cells with Permixon® demonstrated no damage to the nuclear membrane, no cytoplasmic lipid accumulation and no organelle disruption (fig. 2, A-J). In short there were no morphological differences between the Permixon® treated cells and their controls in breast and skin fibroblast as well as in the epididymis, kidney and testes cells.

5α-reductase type I and II activities in Permixon® treated cells. All primary cultures were tested for 5αR-I and 5αR-II isoenzyme activities before and following the addition of the Permixon® (10 μg/ml.) into the incubation medium. Although 5αR-I activity was measured in all cells, the level of activity varied depending on the cell type. The highest activity was found in the prostate epithelial cells (4 ± 0.3 pmol/ DHT/10⁶ cells/30 minutes) with the prostate stromal/fibroblast component showing lower activity (table 1). Non-prostate derived cells demonstrated comparable levels of 5αR-I activity to those measured in the stromal fibroblast (table 1). 5αR-II activity, on the other hand, was confined to the prostate derived cells only (table 1) with prostate epithelial cells demonstrating much higher levels of activity than those measured in the prostate fibroblast cells. Permixon® treatment inhibited the 5αR-I activity of the cultured prostate stromal (p <0.05) and epithelial cells (p <0.05) while no effect was detected on the 5αR-I activity of the non-prostate derived cells. 5αR-II activity in prostate cells was also inhibited significantly by Permixon® (table 1) with the greatest level of inhibition being noted in the prostate epithelial cells (49%; table 1) but measurable inhibition was also detected in the prostate stromal cells (16%; table 1).

The effects of Permixon® on the induction of apoptosis on primary cultures from prostate and other organs. Flow cytometric profiles of all the primary cultures incorporated in the present study revealed only those peaks typical of cell cycling. However when prostate epithelial and stromal/fibroblast cells were treated with Permixon® (10 μg/ml.; 24 hours) a sub G1 apoptosis peak comprising nearly 35% of the
epithelial cell population and 12% of the fibroblast cells was detected by flow cytometry (fig. 3, B). Additionally, a substantial increase in the amount of debris was apparent, consistent with the cell lyses that occurs in the end stage of apoptotic cell death. No change in the percentage of mitotic cells was detected. Similar results were also obtained with skin fibroblast primary cultures where Permixon® induced mild apoptosis (approximately 3% of the cells; fig. 3, B). However all other primary cultures were resistant to the Permixon® which failed to induce apoptosis in these cells thus confirming the morphological studies. These results are summarized in fig. 3, A, B.

DISCUSSION

The n-hexane lipido/sterolic extract of the dwarf palm tree (*Serenoa repens*) is commonly used as a first line treatment for patients with symptoms of outflow tract obstruction due to BPH. 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 5αR. However as previously reported, the method by which Permixon® inhibits 5αR is unclear. Therefore the present investigation was undertaken initially to examine the effect of Permixon® on cells derived from human prostate and non-prostatic organs with a view of elucidating the mechanism of action of the drug and determining its specificity toward the prostate.

This study demonstrated that Permixon® induced a variety of morphological as well as biochemical changes on prostate cells but these were not observed in primary cells cultured from other organs. Most notable was the absence of an effect on the 5αR activity of the non-prostate derived cells underlying the possibility that the inhibitory action of the drug on prostate 5αR is not that of a competitive inhibitor but may be mediated through an intermediate process. Prostate 5αR is strongly bound to the lipid environment of the nuclear membrane. Attempts to disrupt the membrane lead to the inactivation of the enzyme, but this could be reinstated following replacement of the enzyme’s natural niche with exogenous lipids. Studies on phospholipids in human BPH demonstrated that the fatty acid composition of the gland differed not only from patient to patient but also within the same gland and between the epithelium and stroma. Significantly the 5αR activity of the prostate gland appears to be modulated by changes in the fatty acid concentration of the phospholipids. This might also account for the mode of action of the Permixon® which acts selectively on the fatty acid constituent of the prostate nuclear membranes to the exclusion of the other cells. In support of this view are the electron microscopy observations showing the efficacy of Permixon to disrupt the intracellular nuclear membranes of the prostate derived cells but with no similar effect on breast, skin, epididymis, testis & kidneys primary cultures. This is an interesting possibility that clearly needs further investigation. Interestingly, similar observations have been made on LNCaP cells, where intra-cellular damage was observed following incubation with Permixon®. However, the impact of the nuclear membrane disruption on the 5αR activity of the LNCaP cells was not assessed in the earlier study.

In a similar fashion, the data outlined in this document highlights the capacity of Permixon to induce apoptosis in the prostate to the exclusion of the other cells with the exception of skin fibroblasts, which demonstrated a slight increase in apoptotic index following treatment with the drug. The outcome of these experiments is in agreement with the other data in this study and lends some support to earlier reports showing that certain components of Permixon® are selec-

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**FIG. 1.** Electron micrographs of primary cultures of prostate stroma/fibroblast and epithelial cells before and after treatment for 2 days with Permixon® (10 μg/ml.). A, lipid droplets in the cytoplasm and condensation of the chromatin in the nucleus of the treated epithelial cells (original magnification, ×5292). B, control epithelium with round nucleus and microvilli (original magnification, ×7854). C, elongated nuclei, high level of Golgi apparatus and microvilli associated with stroma control (original magnification, ×20520) contrasts with D, general disruption observed in the Permixon® treated stroma/fibroblast (1 day; original magnification, ×7914).
tively concentrated in the prostate to the exclusion of other organs, including the liver. This selectivity is possibly associated with the lipid composition of the prostate cell nuclear membranes and the fact that Permixon® contains fatty acids which could modify the nuclear membrane environment. This might also account for the first demonstration that exposure to Permixon® induces increases in the apoptotic index of the prostate fibromuscular stroma and epithelial cells. While the induction of apoptosis was greater in epithelial cells, nonetheless our data confirmed the earlier observations that fibromuscular stroma previously believed to be strongly resistant to cell death can be induced to undergo apoptosis.

The results reported herein suggest a novel method of enzyme inhibition by Permixon® and demonstrate specificity of the drug for prostatic cells. This hypothesis would also explain the lack of effect on the apoptotic index of Permixon® on non-prostate derived cells. As Permixon® appears to target prostate-derived cells and damage the intra-cellular membranes of these cells it is conceivable that the damage results in death of the cell. Since non-prostate derived cells do not appear to be susceptible to Permixon®, and display no intra-cellular membrane damage, these cells will, in turn, show no increase in apoptosis following Permixon® treatment.

Although we have demonstrated a level of specificity for prostate cells by Permixon®, further studies on the phospholipid composition of the nuclear cell membrane and the af-

![Fig. 2. Electron micrographs of primary cultures of skin, breast, testis, epididymis and Leydig cells before and after treatment for 2 days with Permixon® (10 μg/ml). There were no morphological differences between the Permixon treated cells and their controls. a, Epididymis treated (original magnification, ×28080). b, epididymis control (magnification, ×9968). c, testes treated (original magnification, ×20736). d, testes control (original magnification, ×20736). e, skin fibroblast treated (original magnification, ×9720). f, skin fibroblast control (original magnification, ×9720). g, breast fibroblast treated (original magnification, ×5292). h, breast fibroblast control (original magnification, ×5292). i, kidney cells treated (original magnification, ×5554). j, kidney cells control (original magnification, ×5554).]
finity of the drug components for these constituents may elucidate the precise mechanism(s) responsible for the observations described in this study.

CONCLUSIONS

This study highlighted the selectivity of the action of Permixon® for prostate cells as demonstrated by the damage to the intra-cellular membrane of these cells following treatment with the drug. The morphological changes in the prostate are accompanied by an increase in the apoptotic index along with an inhibition in the activity of the nuclear membrane bound 5αR isoenzymes. No similar changes were observed in any of the other cells under investigation. Nuclear membrane disruption could be the underlying mechanism for the 5αR-I and 5αR-II inhibitory effect of Permixon® in prostate cells.

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REFERENCES

EFFECTS OF PERMIXON® ON PROSTATE MORPHOLOGY AND APOPTOSIS


