PRECLINICAL AND CLINICAL INVESTIGATIONS AS AN APPROACH TO RATIONAL PHYTOTHERAPY IN PROSTATE DISEASES

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List of abbreviations

ALAT	Alanine aminotransferase
AR	Androgen receptor
ASAT	Aspartate aminotransferase
B.A.D.	Background aggregates and debris
BP	Blood pressure
BPH	Benign prostatic hyperplasia
BOO	Bladder outlet obstruction
BSA	Bovine serum albumin
COX	Cyclooxygenase
CV	Crystal violet
DSB	Diastolic blood pressure
DHT	5α-Dihydrotestosteron
DMSO	Dimethylsulfoxide
eCRF	Electronic case report form
EGF	Epithelial growth factor
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorter
FA	Fatty acids
FBS	Fetal bovine serum
bFGF	Basic fibroblast growth factor
HPLC	High performance liquid chromatography
IPSS	International prostate symptome score
IIEF	International index of erectil function
LTB_4	Leucotriene B_4
LDH	Lactat dehydrogenase
LOX	Lipooxygenase
LUTS	Lower urinary tract symptoms
O.D.	Optical density
NS	Non-significant
PBS	Phosphate-buffered saline
PCA	Prostate carcinoma
PGE_2	Prostaglandin E_2
P	Prostate volume
PS	Penicillin/streptomycin
PSA	Prostate specific antigen
Q_{ave}	Mean urinary flow rate
Q_{max}	Peak urinary flow rate
QoL	Quality-of-life index

R	Postvoid residual urine volume
SBP	Systolic blood pressure
S.repens	Serenoa repens
SRE	Serenoa repens extract
SV40T	SV40 large T (antigen)
VAC	Vitex agnus-castus extract
VACF	Vitex agnus-castus fruit extract
VACL	Vitex agnus-castus leaf extract

Summary

Phytotherapeuticals have gained widespread usage in the treatment of symptomatic benign prostatic hyperplasia (BPH). Among these phytotherapeutics, the most popular and extensively studied are extracts of the dried ripe fruit of *Serenoa repens* (SRE). The commercially available hydroethanolic (96%) SRE, Me180, is already used as a medical treatment of symptomatic BPH. However, this extract was so far neither investigated in vitro nor in vivo. The other extracts tested herein, derived from *Vitex agnus-castus* (VAC), are so far not described to be used for treatment of prostatic diseases or tested on prostate cells.

In a first part of the present studies, an in vitro screening system was established, representative for different prostate disease stages. BPH and prostate cancer (PCA) are multifactorial disease processes. Both diseases are age-related proliferative disorders of the human prostate and with disturbed homeostasis in both conditions by upsetting the balance between prostate cell proliferation and apoptosis. Thus, the inhibition of proliferation and the reversion of the imbalance of homoeostasis is a desired therapeutic option in both diseases.

Three different cell lines (BPH-1, LNCaP, PC-3) were chosen, representing different disease stages. To evaluate the potential effects of selected plant extracts in proliferative prostate diseases, several methodological approaches were used: Crystal violet staining and WST-8 (tetrazolium based assay) were conducted to establish the effects on cell proliferation and viability. To further elucidate the mechanisms of cell death, FACScan analysis was performed to detect effects on cell cycle distribution and apoptosis. Apoptosis was further investigated by co-treatment of the cells with a pan-caspase inhibitor Z-VAD-fmk and by DNA fragmentation assays. In addition, cytotoxicity was determined by measurement of lactate dehydrogenase (LDH) in cell supernatants.

Proliferation of BPH-1 and PC-3 cells was modestly inhibited by *Serenoa repens* extract Me180 in a dose-dependent manner with IC_{50} values of 100µg/ml, whereas in androgen-responsive LNCaP cells, lower IC50 values of 35µg/ml, indicated a possible mechanism through androgen-receptors. Me180 exerted similar effects in vitro as other SRE's previously investigated. Additional SRE's, which varied with respect to ripeness conditions of the raw material and the choice of solvents (hexane

or ethanol) displayed no crucial differences in potency of growth inhibitory activities. Moreover, Me180 exhibited a dose-dependent apoptotic effect in LNCaP cells, with additional low cytotoxic effects. A low apoptotic effect was found in BPH-1 cells without a cytotoxic effect, whereas PC-3 cells were almost insensitive to the Me180 treatment.

In addition, using the same methodology, these preclinical investigations were the first to examine the effects of VAC fruit and leaf extracts on prostate cell proliferation. The two different VAC's were found to be effective inhibitors in all three cell lines (with IC_{50} values < 10µg/ml after exposure to fruit extract and < 20µg/ml for leaf extract, respectively) inducing apoptosis, and a low cytotoxic effect. Our new results suggest that extracts of VAC, by modulating the cell cycle and the apoptotic machinery, may possess a potential for development as an agent for prevention and/or therapy of benign and malign prostatic hyperplastic diseases. Additional studies are however required to further clarify their mechanisms of action and to investigate their effects in vivo.

The in vitro data indicate that the chosen screening model system is suitable for the detection of growth inhibitory activities of plant extracts in human prostate cell lines and may help to find new phytotherapeuticals for this field of indication.

The objective of the second part was a clinical pilot study to compare the therapeutical effects, tolerability and safety of treatment with *Serenoa repens* extract Me180 (SabCaps®) versus tamsulosin (Pradif®) in patients with obstructive BPH. Due to stringent exclusion criteria and hence difficulties in patient recruitment, the planned patient number of 70 could not be achieved within a reasonable time for the thesis. Only 19 patients, aged 50-79 years, with obstructive BPH (mean Qmax < 10ml/s for a voiding volume of 150ml or assessed by flow-pressure study) were therefore included in the study. Patients were randomised with either 320mg Me180 or Tamsulosin 0.4mg, given over a scheduled treatment period of 26 weeks in a double-blind manner. The primary outcome measures were the change in peak urinary flow rate (Q_{max}) and International Prostate Symptom Score (IPSS) with quality-of-life score during the treatment. The secondary outcome measures included average urinary flow rate (Qave), post-void residual urine volume and prostate volume, assessed by transabdominal ultrasound. In addition, the International Index of Erectile Function (IIEF) score was determined and adverse events were recorded.

No significant differences were found in baseline parameters between the two treatment groups. The treatment over 26 weeks was completed by 18 patients. In the tamsulosin group, one patient withdrew from the study because of continuous dizziness. There was no significant difference between the two treatments over the 26 weeks of the study with respect to IPSS, Q_{max}, Q_{ave}, residual urine, prostate volume, and IIEF. During this pilot study all participants had some improvements in their symptoms of BPH, but there was no significant difference in the beneficial effect of either medication over the 26-weeks treatment time. With respect to the overall incidence of adverse effects, Me180 was found significantly superior to the tamsulosin group ($p = 0.013$).

The present data describe for the first time clinical results obtained with Me180. Based on this pilot study, future clinical trials with larger patient numbers and longer periods of treatment will be needed to assert if Me180 is an efficacious alternative medical therapy for men with obstructive BPH.

I INTRODUCTION AND AIM OF THIS THESIS

The popularity of phytotherapy is at an all-time peak. Many patients are searching for 'natural' alternatives or complements to conventional drug therapy. Herbal pharmaceuticals are generally promoted as being effective for chronic diseases and devoid of adverse effects. However, plant extracts, like any other drugs, may exhibit side-effects and interactions with other therapeutics but unfortunately the majority of the herbal drugs have not been systematically examined. Phytotherapeuticals should be tested in clinical studies with reference (placebo or/ and synthetic) medications that are therapeutically equivalent to single compounds. Medical-scientific standards in clinical studies (Good Clinical Practice, GCP) with particular indications have to prove their efficacy and safety. Moreover, phytotherapeuticals must be of high pharmaceutical quality (Good Manufacturing Practice, GMP). Thus, based on this knowledge, the rational phytotherapy can be considered as a treatment alternative.

Benign prostate hyperplasia (BPH) is a non-malignant progressive enlargement of the prostate, which can result in bothersome lower urinary tract symptoms (LUTS). The frequency of prostate enlargement and associated symptoms increase with age (Berry et al 1984). Prostate cancer (PCA) is the most frequently diagnosed cancer in men in Western countries and the second leading cause of cancer-related deaths in men (Hsing 2000). Although there is no evidence for an association of these two proliferative prostatic diseases (Bostwick 1998), both diseases display an age related increase in prevalence. With increasing mean life expectancy, the population of men being affected by these diseases will expand and the importance of finding effective and well tolerated preventive and therapeutic agents rises.

Phytotherapeuticals have gained widespread usage in the treatment of symptomatic BPH. Among these phytotherapeutics, the most popular and extensively studied are extracts of the dried ripe fruit of *Serenoa repens* (Lowe & Fagelman 2002). Despite its popularity as a medication, its efficacy and safety for the treatment of symptomatic BPH and the possible mechanism of action are still not fully clarified. Although numerous in vitro experiments have been conducted to determine their possible mechanisms of action, it is uncertain which of the actions demonstrated in vitro might be responsible for clinical responses in patients. Only a few randomized clinical trials that meet standard criteria of evidence-based medicine but relatively short follow-up

times (up to 1 year) were conducted (Carraro et al 1996; Debruyne et al 2002; Willetts et al 2003). Thus, the extent of their therapeutic potential remains uncertain. Moreover, most of these trials were conducted with the same *Serenoa repens* hexane extract (Permixon®). Most of the plant extracts are unique: (1) because of natural variability of different plant sources, the plants are not identical, (2) the extraction processes used by the various manufacturers are frequently different, and (3) various extraction agents are used for the process, all of which makes comparison of the preparations difficult. Therefore, each extract must be studied individually and the production of each of these extracts has to be standardised.

In contrast to established knowledge with *Serenoa repens* extracts, extracts of *Vitex agnus-castus* are so far not described to be used in prostatic diseases. VAC fruits extracts were reported to have a beneficial effect on disorders related to hyperprolactinaemia such as cycle disorders, luteal phase defect, mastodynia and premenstrual syndrome (Wuttke et al 2003). Recently, a VACF was shown to exhibit anti-tumour activities in different human cancer cell lines (Ohyama et al 2003). Its effects on human prostate epithelial cells have so far not been investigated.

Against this background the objectives of the present studies were as follows:

PRECLINICAL INVESTIGATION

a) Establishment of a representative in vitro cell culture system using three different human prostate cell lines indicative of different disease stages. A variety of plant extracts were investigated for specific prostate cell functions: cell viability, cell proliferation, cell cycle distribution, apoptosis and cytotoxic effects.

b) The commercially available hydroethanolic (96%) *Serenoa repens* extract Me 180 is already used as a medical treatment of symptomatic BPH. However, to our knowledge, this extract was so far neither investigated in vitro nor in vivo. Therefore, this extract was included in the in vitro test model system.

c) With the aim to investigate the role of the extract quality of *Serenoa repens* we further examined the influence of extracts from different ripening conditions and different extraction agents on prostate cell growth.

d) *Vitex agnus-castus fruit extract* was examined for the first time on this established prostate in vitro test model system. Moreover, the effects of a selected fruit extract were compared with a leaf extract.

CLINICAL INVESTIGATIONS

In a second part, a controlled randomised double-blind pilot study was conducted to compare the therapeutical effects and safety of the commercial *Serenoa repens* (Me180, SabCaps®) extract with Tamsulosin (Pradif®) in patients with obstructive BPH. As mentioned before, although this extract is already commercially available, it has so far not been investigated in patients with BPH. The outcome of the present pilot study is intended to form the basis for the design of an extended study with more patients to assess the efficacy of the extract over a longer treatment period.

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II Background

1 Proliferative prostate diseases

Benign prostatic hyperplasia (BPH) and prostate cancer (PCA) get manifest in men beyond the age of 50. Both diseases display an age related increase in prevalence, require androgens for growth and development, and may respond to androgen deprivation treatment. In both conditions, there is an imbalance between cell proliferation and cell death by apoptosis, resulting in increased cell number (Figure II-1) (Thompson & Yang 2000).

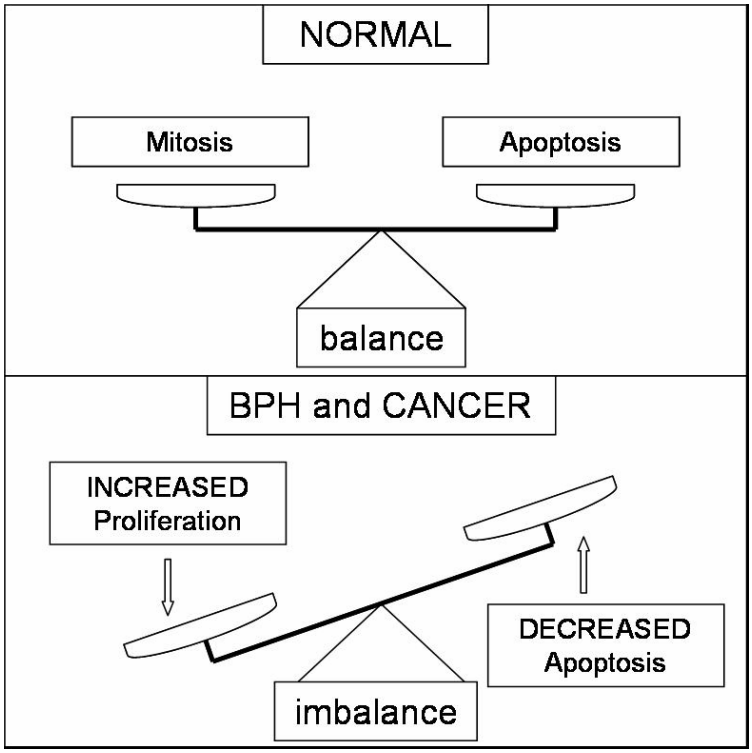

Figure II- 1 The balance between cell proliferation and cell death

However, BPH and PCA are primarily entities of two different areas of the prostate; the transition zone has a high incidence of BPH and a low incidence of carcinoma, whereas the peripheral zone has a high incidence of carcinoma and low incidence of BPH (De Marzo et al 1999). Thus, there is no evidence that PCA is associated with BPH and BPH does not appear to directly progress to carcinoma (Bostwick et al 1992; Bostwick 1998).

1.1 Benign prostatic hyperplasia (BPH)

Benign prostatic hyperplasia (BPH), as the name implies, is a benign disorder that develops predominantly adjacent to the urethra in the transition zone of the prostate. BPH presents histologically as a mixture of epithelial (glandular) and stromal (smooth muscle and connective tissue) hyperplasia. Despite the well-characterized histology associated with BPH, little is known about the underlying aetiology of the disease and the precise factors responsible for the pathogenesis of BPH are incompletely known (Walden et al 1998). However, the metabolism and action of steroid hormones appears to be strongly associated with the proliferation of epithelial and stromal prostate cells and thus with the development of BPH.

The following findings appear to play a crucial role in the development of BPH:

1. The prostate is androgen-dependent and a source of testosterone is necessary for its growth. However, the intraprostatic androgen 5α-dihydrotestosterone (DHT), a metabolite of testosterone converted by the 5α-reductase, preferentially binds to the androgen receptor. DHT is necessary for prostate growth and appears to play an important role in regulation of gene expression (Griffiths et al 1998).
2. As men get older, the androgen/ estrogen ratio falls due to the rise in estrogen production and evidence has been accumulating that estrogens (particularly the testosterone/oestrogen ratio) are important regulators, particularly for prostatic stromal development (Farnsworth 1999).
3. Prostate cell proliferation, differentiation and apoptosis are regulated by the integral network formed by prostatic epithelial and stromal cells and the extracellular matrix for paracrine and autocrine communication by intrinsic factors. Stromal-epithelial interactions are mediated by steroid hormones (androgen, oestrogen) action and steroids also regulate the expression of the EGF, IGF and TGF-α families of growth factors and receptors (Brown & Lee 2001).
4. It has been demonstrated that infiltration of the prostate by inflammatory cells is one of the aetiologic factors involved in the development of BPH. These inflammatory cell types, such as polymorphonuclear neutrophils (PMNs), produce chemotactic mediators and contribute to the development of the disease. (Theyer et al 1992)

Histological evidence of BPH is found with increasing frequency in elderly men. Berry and co-workers reviewed five autopsy series and found that BPH is not seen before the fourth decade. Histological BPH is found in 40% of men aged 51-60 and 90% of men over 80 (Figure II-2) (Berry et al 1984).

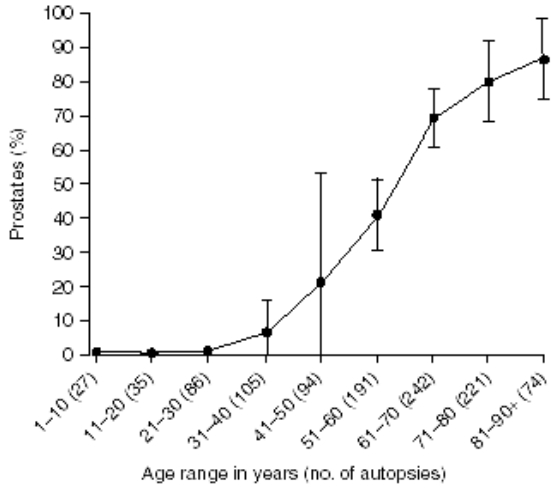

Figure II- 2 Proportion of prostates with histopathological benign prostatic hyperplasia (BPH) at autopsy versus age. Mean ± SE. Reprinted from (Berry et al 1984)

Strictly speaking the terminus 'benign prostate hyperplasia' (BPH) describes exclusively the histological diagnosis. However, in general BPH is used undifferentiated as a synonym for a complex combination of lower urinary tract symptoms (LUTS). LUTS are usually, but not exclusively associated with benign prostatic hyperplasia (BPH). LUTS can be irritative or obstructive. The former occur during the bladder emptying phase and include hesitancy, decreased stream, feeling of incomplete emptying, straining to void, intermittency, postvoid dribbling, and urinary retention. The latter occur during the storing phase and include daytime frequency, nocturia, urgency and urge incontinence associated with involuntary detrusor contractions (Barry & Roehrborn 2001). The prevalence of LUTS related to BPH depends on the definition used. However, it is obvious that LUTS have a significant negative impact on the quality of life of patients.

The relationship between BPH and LUTS is complex, because not all men with histological evidence of BPH will develop LUTS. In addition, LUTS are neither specific for BPH, other conditions in the lower urinary tract and elsewhere may be causative. As the prostate gland enlarges, it can cause both obstructive and irritative symptoms; however, the size of the prostate gland is not predictive of the symptoms that patients experience. Volume and form of the prostate can be measured

precisely, but are only of secondary importance for the medical condition. It seems undisputable that the severity of the symptoms of BPH does not correlate with the volume of the prostate. Significant for the characteristics of the symptoms is the direction of increase of the prostate (Barry et al 1993).

Although LUTS are suggestive of infravesical obstruction, they are not specific to BPH and therefore certain diagnostic tests may be necessary to confirm obstruction. There is universal agreement that the urethra is a distensible elastic tube with a flow controlling zone normally located in the membranous urethra. With the development of prostatic obstruction the flow controlling zone moves into the prostatic urethra. The enlarging prostate may or may not cause an infravesical obstruction. If obstruction develops, it is thought to be partly due to the anatomical presence of the enlarged prostate (mechanical or static obstruction) and partly due to the tonus exerted by the smooth muscle in the prostate and in the bladder neck (dynamic obstruction). The increased urethral resistance prevents the detrusor muscle from rapidly expelling urine which again gives rise to a higher driving pressure (Hald et al 1998).

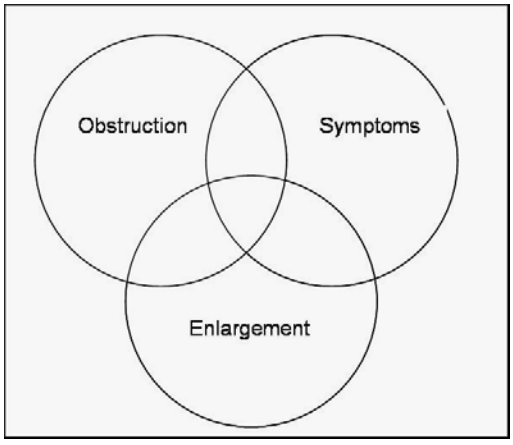

Figure II- 3 Basic features of clinical BPH: obstruction, symptoms and hyperplasia. They overlap but are independent variables determining the nature of the clinical situation (Hald 1989).

Taken together, these three mentioned main aspects, symptomatology, prostate enlargement and infravesical obstruction (shown in Figure II-3) determine the clinical picture of BPH-patients (Hald et al 1998) and they are variable from one patient to another. It is therefore of importance that the condition referred to as clinical BPH be defined, especially in clinical trials. Various different symptom questionnaires and inclusion criteria complicate the comparison of trials in this indication. Therefore,

these basic features of BPH should be clearly defined in clinical trials and inclusion and exclusion criteria should be applied according to the current recommendations of the International Consultation on BPH.

1.2 Treatment options for symptomatic BPH

The main objectives of treatment for LUTS suggestive of BPH, are to provide fast and sustained relief of bothersome symptoms, to improve quality of life and to control disease progression (Schulman 2003). Current treatment options include 'watchful waiting', phytotherapeuticals, 5α-reductase inhibitors, α_1-blockers, surgical intervention. For some decades, transurethral resection of the prostate (TURP) has been the gold standard treatment. However, the incidence of complications such as blood loss, urinary tract infections, urethral stenosis, incontinence, impotence, and the need for re-intervention is clinically significant after prostatic surgery (Kaplan et al 1995). Moreover, most patients with symptomatic BPH reportedly choose less aggressive interventions than invasive surgery, although more active than watchful waiting.

Currently, α-blockers are becoming first-line drug therapy compared with the 5α-reductase inhibitors and phytotherapy (Chapple 2001). The prostate contains stromal tissue with a smooth muscle component. α-blockers inhibit α_1-adrenergic receptors and have a clinical effect by reducing the sympathetic tone, thereby relaxing smooth muscle in the hyperplastic prostate and relieving urinary obstruction. Several investigations document significantly more α_1- adrenergic receptors in hypertrophic prostate tissue than in normal tissue (Yamada et al 1987) and blocking urethral α_1-adreno-receptors causes the prostate urethra to relax (Chapple 2001). The α_{1A}-receptor appears to be the subtype mainly mediating prostatic and bladder smooth muscle tone, the dynamic component of bladder outflow obstruction (Walden et al 1999). Unlike the other common α-blocking agents that are not selective for this subtype (doxazosin, alfuzosin and terazosin), tamsulosin possesses high α_{1A}-receptor affinity. Common adverse events associated with α_1- adrenergic receptor blockers are dizziness, asthenia, tachycardia/palpitation, orthostatic hypotension, somnolence or abnormal ejaculation. Because of its 'prostate-selectivity' tamsulosin may improve urinary symptoms and flow with fewer adverse effects (Wilt et al 2003).

1.3 Prostate Cancer (PCA)

Prostate cancer (PCA) is the most frequently diagnosed cancer in men in Western countries and the second leading cause of cancer-related deaths in men (Hsing 2000). It is a very well-known fact that prostate cancer and male sex hormones are strongly interrelated. Prostate cancers are well known to progress from hormone sensitive to hormone therapy-resistant terminal states. Treatment of advanced prostate cancer often involves either surgical gonadectomy or drugs that inhibit androgen production and action. Unfortunately, prostate cancer often metastasises to lymph nodes, bone, and other distant sites. Metastatic prostate cancer initially responds to androgen withdrawal therapy, but tends to become androgen-independent subsequent to hormonal treatment, so that many patients fail this therapy (Shaffer & Scher 2003; So et al 2003).

One of the goals of current research on PCA is the identification of new agents that would prevent and/or slow down the development of this disease. As the incidence of prostate cancer has risen in public awareness and in the statistical tables of cancer incidence, so aging men have become increasingly more interested in what they can do to preserve their health and avoid diseases and prevent their progression. One of the reasons to evaluate phytotherapeutic therapies is the possibility that particular dietary factors may be responsible for the internationally wide variations that exist in the prevalence of and mortality from prostate cancer. In particular, Asian men are at significantly lower risk than their North American and Western European counterparts (Pisani et al 2002). Asian men who migrate to North America have an increased mortality from prostate cancer compared with men in their native countries. This implies that environmental factors, rather than genetics alone, may contribute to the risk of prostate cancer (Denis et al 1999).

2 Selected plant extracts

Phytotherapeuticals have gained widespread usage in the treatment of symptomatic BPH, and there are numerous plant extracts that are widely used in this indication (see Table II-1) (Weiss 1999).

Table II- 1 Origin of plant extracts currently used for symptomatic BPH

Species	Common name	Part
Serenoa repens	Saw palmetto (Sägezahnpalme)	Fruits
Hypoxis rooperi	South African star grass (Südafrikanisches Sternengras)	Roots
Pygeum africanum	African plum tree (Afrikanischer Pflaumenbaum)	Bark
Urtica dioica	Stinging nettle (Brennessel)	Roots
Secale cereale	Rye pollen (Roggenpollen)	Pollen
Cucurbita pepo	Pumpkin (Kürbis)	Seeds

Most of these plant extracts have not been studied in great detail and there is a lack of information on their mode of action. Among these phytotherapeutics, the most popular and extensively studied are extracts of the dried ripe fruit of *Serenoa repens* (Lowe & Fagelman 2002).

2.1 Serenoa repens

Key Points about *Serenoa repens*

Species name:	*Serenoa repens* (Bartr.) Small Syn. : Sabal serrulata (Michaux) Nutt. ex Schultes
Family:	Arecaceae (Palmae)
Habitat:	*Serenoa repens* is native to the coastal regions of the southern states of the United States from South Carolina to Florida, Southern California, Southern Europe and North Africa.
Drug:	Sabal fructus Partially ripened, dried fruits of *Serenoa repens* (Bartr.) Small
Principle constituents:	Free and esterified fatty acids Long-chain saturated and unsaturated alcohols Phytosterols Flavonoids Polysaccharides
Indication:	Symptomatic treatment of lower urinary tract symptoms (LUTS) Secondary to mild and moderate benign prostatic hyperplasia
Postulated mechanisms of action:	5α-reductase inhibition (type1 and 2) Anti-androgenic Anti-estrogenic Anti-proliferative Anti-inflammatory α_1-adrenergic blocking activity
Dosage:	Varies; most studies have used 320mg once daily or 160mg twice daily of hexane, hydroethanolic or supercritical CO_2 extract
Adverse effects:	Mild gastrointestinal distress; infrequent
Contraindication:	No known
Drug interactions:	No known drug interactions, except from one case report of severe intraoperative haemorrhage in a patient who was taking *Serenoa repens.*(Cheema et al 2001)

Reviewed in (Wilt et al 1998; Boyle et al 2000; Dreikorn 2002)

Serenoa repens extracts (SRE) are the most popular phytotherapeutical medication used in the treatment of LUTS suggestive of BPH. However, the complete mode of action of the extracts is still under investigation. The effects of SRE's were investigated and explained through a multifactorial mechanism including an anti-androgenic, anti-estrogenic, and growth inhibitory action, and to interfere with prolactin receptor signal-transduction, to exhibit anti-inflammatory action and to relax prostatic smooth muscle:

Several SREs were found to inhibit the 5α-reductase in vitro in fibroblasts and in homogenates of human BPH tissue (Weisser et al 1996) and in stromal and epithelial cells from human BPH, either separated (Delos et al 1995) or co-cultured (Bayne et al 1999). SRE has been shown to act as an inhibitor for both isoforms of 5α-reductase (type 1 and type 2) in a baculovirus-directed insect cell expression system (Delos et al 1994; Iehlé et al 1995; Raynaud et al 2002). In all these models, SRE was shown to be a non-competitive inhibitor of 5α-reductase, suggesting that the mode of action is rather a modulation of the activity by modification of the lipid environment of the enzyme than an interaction at its catalytic site (Delos et al 1994; Bayne et al 1999). Moreover, there were some conflicting results with regard to the competitive binding affinities of SRE to androgen receptors (Sultan et al 1984; Ravenna et al 1996; Bayne et al 1999).

It was shown in an earlier study, that androgen and estrogen receptors became localized in the cytoplasm of the prostate cells following treatment with SRE (Di Silverio et al 1992). It was suggested that this localization of the receptors may also be due to the receptors leaking out of the nucleus into the cytoplasm, a process facilitated by the SRE-damaged nuclear membrane. In epithelial and fibroblast cell cultures derived from BPH patients, SRE also inhibits 17β-hydroxysteroid dehydrogenase, the enzyme responsible for converting androstenedione into testosterone (Delos et al 1995).

Another postulated important action of SRE is the inhibition of the proliferation of human prostate cells in vitro induced by basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Paubert-Braquet et al 1998). Furthermore, SRE also induced a significant reduction in dihydrotestosterone (DHT) and EGF concentrations in human BPH following 3-month treatments (Di Silverio et al 1998). In addition, SRE was shown to have several other effects and actions 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 (Paubert-Braquet et al 1998), and inhibition of prolactin receptor signal transduction in Chinese hamster ovary (CHO) cells (Vacher et al 1995). The latter effect resulted in reduced K^+channel conductance and a reduction in the activity of protein kinase C (PKC). It was moreover demonstrated that in patients treated with SRE, the extract induces apoptosis in epithelium and in stroma (Vacherot et al 2000), and mixed cell death of apoptosis and necrosis in LNCaP cells, an androgen-responsive human prostate cell line (Iguchi et al 2001). Additionally, the extract was shown to inhibit urokinase-type plasminogen activator leading to suppression of cell invasion in androgen-unresponsive human prostate cancer cells (PC-3) (Ishii et al 2001)

There is also evidence that SRE may act as an anti-inflammatory agent inhibiting both phospholipase A2 and the oxidative enzymes responsible for the synthesis of the pro-inflammatory eicosanoids (Paubert-Braquet et al 1997), inhibiting both cyclooxygenase and lipoxygenase (Wagner et al 1981; Breu et al 1992). In a recent pilot study the anti-inflammatory activity of SRE was underlined by histological investigations after treatment with the extract (Vela Navarrete et al 2003).

Numerous clinical trials with SRE have been published. Most are uncontrolled open-label studies, which provide little useful information for determining the efficacy of these phytotherapies. However, two randomised, double-blind studies have shown an *Serenoa repens* hexane extract (Permixon®) to be as effective as the uro-selective α_1-adrenergic blocker, tamsulosin and the 5α-reductase inhibitor, finasteride, in achieving subjective and objective improvements of symptomatic BPH (Carraro et al 1996; Debruyne et al 2002) after treatment of 6 months and 12 months, respectively. A more recent urodynamic trial of 75 patients treated with SRE or placebo showed improvement in urodynamic parameters (peak urinary flow, detrusor pressure at maximum flow, residual volume, and opening detrusor pressure) after a 9-week-treatment with extract compared to placebo (Al-Shukri et al 2000). However, in a recent double-blind placebo-controlled randomized trial, no significant difference between the SRE and placebo was shown over the 12 weeks of the study in the symptom score (IPSS) and peak urinary flow rate (Willetts et al 2003). Thus, the efficacy of SRE for the treatment of symptomatic BPH needs to be investigated in further clinical trials.

2.2 Vitex agnus-castus

Key Points about Vitex agnus-castus (German Commission E Monograph)

Species name:	Vitex agnus-castus L.
Family:	Verbenaceae
Habitat:	Vitex agnus-castus is native to Mediterranean regions, Asia and South America.
Drug:	Agni casti fructus Ripe, dried fruits of Vitex agnus castus L.
Principle constituents:	Iridoid glycosides (Agnusid, aucubin) Aetheric oil Diterpenes (rotundifuran, vitexilacton, 6B,7B-DHLD) Flavonoids Fatty acids (linolic and linoleic acid) Sterols controversially discussed
Indication:	Premenstrual complaints, irregularities of the menstrual cycle, mastodynia.
Mechanism of action:	Dopaminergic action Inhibition of prolactin secretion action
Dosage:	Aqueous-alcoholic extracts corresponding to 30-40mg of the drug
Adverse effects:	Occasional occurrence of itching, urticarial exanthemas
Contraindication:	None known
Drug interactions:	Interactions are unknown

Vitex agnus-castus (VAC) is belonging to the Verbenaceae family, which grows naturally and is common in Southern Europe, in the Middle East and in South America. Its ripened fruits have a long history of use as a hormone balancing remedy and a legendary remedy to help subdue excited libidos among those who would have to remain chaste, like monks.

More recently, *Vitex agnus-castus* fruit extract (VACF) was reported to have a beneficial effect on disorders related to hyperprolactinemia such as premenstrual syndrome, cycle disorders, luteal phase defect, and mastodynia (Schellenberg 2001; Wuttke et al 2003). Several reports document prolactin-suppressive activities from VACF: Prolactin release from cultivated rat pituitary cells was suppressed after treatment with VACF (Jarry et al 1994). In a double-blind, placebo-controlled clinical trial the extract reduced serum prolactin levels in female patients treated (Wuttke et al 1997). Prolactin synthesis is regulated by the neurotransmitter dopamine (MacLeod 1969). Extracts of VACF were shown to exhibit dopamine binding activity to recombinant dopamine D2 and dopamine D3 receptor protein (Wuttke 1995; Meier et al 2000; Simmen et al 2003). It was therefore proposed that the prolactin suppressive action of VACF is due to a dopaminergic action (Wuttke et al 2003). Beyond the dopaminergic effect additional pharmacological actions via μ- and k-opioid receptors are suggested (Bruggisser et al 1999).

Furthermore, fruit and leave extracts were shown to exhibit estrogenic activity, whereby the leaf extract was shown to be more active than fruit extract (Berger 1998). Investigations on human recombinant estrogen receptor subtype -α and –β revealed a VACF- induced inhibition of both (Liu et al 2001; Wuttke et al 2003).

Beyond this indication field, a VACF was recently shown to exhibit anti-tumour activities in different human cancer cell lines, which included carcinoma cell lines of breast, ovarian, gastric, colon, cervix and lung and was compared with “normal” human fibroblasts (Ohyama et al 2003). Additionally, in a recent diploma thesis, concurrent with the present study, a VACF extract was reported to inhibit the activity of recombinant cyclooxygenases COX-1 and COX-2 and 5-lipooxygenase 5-LOX activity of differentiated promyelocytic leukemia cells HL-60 cells after stimulation with arachidonic acid (Hubacher 2002).

Based on this knowledge the rationale for the selection of *Vitex agnus-castus* extract (VAC) as a potential phytotherapeutical drug for proliferative prostate diseases are forfold:

1. **VACF induced cell growth inhibitory action in several human cell lines**

   A VACF extract was shown to exhibit anti-tumor activities in different human cancer cell lines (Ohyama et al 2003), but its effect on human prostate epithelial cells has so far not been investigated.

2. **Dopaminergic and prolactin secretion inhibiting action of VAC**

   VACF was shown to exert inhibition of prolactin secretion via a dopaminergic action (Wuttke et al 2003). Prolactin was reported to stimulate the proliferation and the differentiation of prostate cells. It was shown that prolactin acts in synergy with androgens either by enhancing the testosterone effect or by increasing the number of cytosolic and nuclear androgen receptors (reviewed by Reiter et al 1999). Therefore, inhibition of prolactin secretion by VACF may exhibit a beneficial effect in both proliferative prostatic diseases.

3. **COX-1, COX-2 and 5-LOX inhibiting action in vitro**

   A VACF was reported to inhibit the activity of recombinant COX-1 and COX-2 and 5-LOX activity of differenciated HL-60 cells after stimulation with arachidonic acid (Hubacher 2002). The prostate is often the target of acute and chronic inflammatory responses (Donovan & Nicholas 1997). These observations may point to a role of inflammatory mediators in the pathogenesis of BPH (Eaton 2003). In addition, long-term chronic inflammation may contribute to carcinogenesis in many organ systems through a postulated mechanism of repetitive tissue damage and regeneration in the presence of reactive phagocyte-derived oxygen and nitrogen species and cytokines (Ames et al 1995). Moreover, it was shown that expression of both COX-1 and particularly COX-2 is increased in human PCA (Kirschenbaum et al 2000), indicating that COX-1 and COX-2 (and/or their prostaglandin products) may play a crucial role in the malignant transformation of the prostate. Therefore, COX-1, COX-2 and 5-

LOX inhibitory properties of VACF may exhibit a beneficial effect in both proliferative prostatic diseases.

4. Selective estrogen receptor action

Estrogen receptor β (ER-β) is preferentially localised in the prostate epithelium (Chang & Prins 1999). ER-β knockout mice exhibit prostatic hyperplasia and this animal model favours an antiproliferative role of ER-β (Weihua et al 2002). In this regard it was proposed that ER-β is a potential target for therapeutical intervention in proliferative prostatic disease. It was shown by Berger that VACF exhibits estrogen activity (Berger 1998). Moreover, Jarry and co-workers reported of a selective action on recombinant estrogen β receptor (Jarry et al 2003). Therefore, estrogen β activity properties of VACF may exhibit a beneficial effect in both proliferative prostatic diseases.

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III PRECLINICAL INVESTIGATIONS

Development of a screening model system and in vitro testing of *Serenoa repens* and *Vitex agnus-castus* extracts for rational use in proliferative prostate disorders

1 Introduction

Benign prostatic hyperplasia (BPH) and prostate cancer (PCA) are multifactorial disease processes, the pathogenesis of which is only poorly understood. However, the pathogenesis of both, BPH and PCA is likely to be related to uncontrolled growth of the prostate gland. Both diseases are age-related proliferative disorders of the human prostate with disturbed homeostasis in both conditions by upsetting the balance between prostate cell proliferation and apoptosis, leading to BPH and PCA, respectively (Figure II-1).Thus, the inhibition of proliferation and the reversion of the imbalance of homoeostasis is a desired therapeutic option. To evaluate the potential effects of selected plant extracts in these common age-related prostatic disorders in elderly men, an in vitro screening system representative for different disease stages was established.

1.1 Overview of the in vitro test system

Figure III-1 gives an overview of the analysis conducted to assess the in vitro effects of selected plant extracts in human prostate cells lines. The chosen assay systems are well accepted tools in preclinical drug development and therefore suitable methods for the evaluation of effects of plant extracts as an approach of rational phytotherapy.

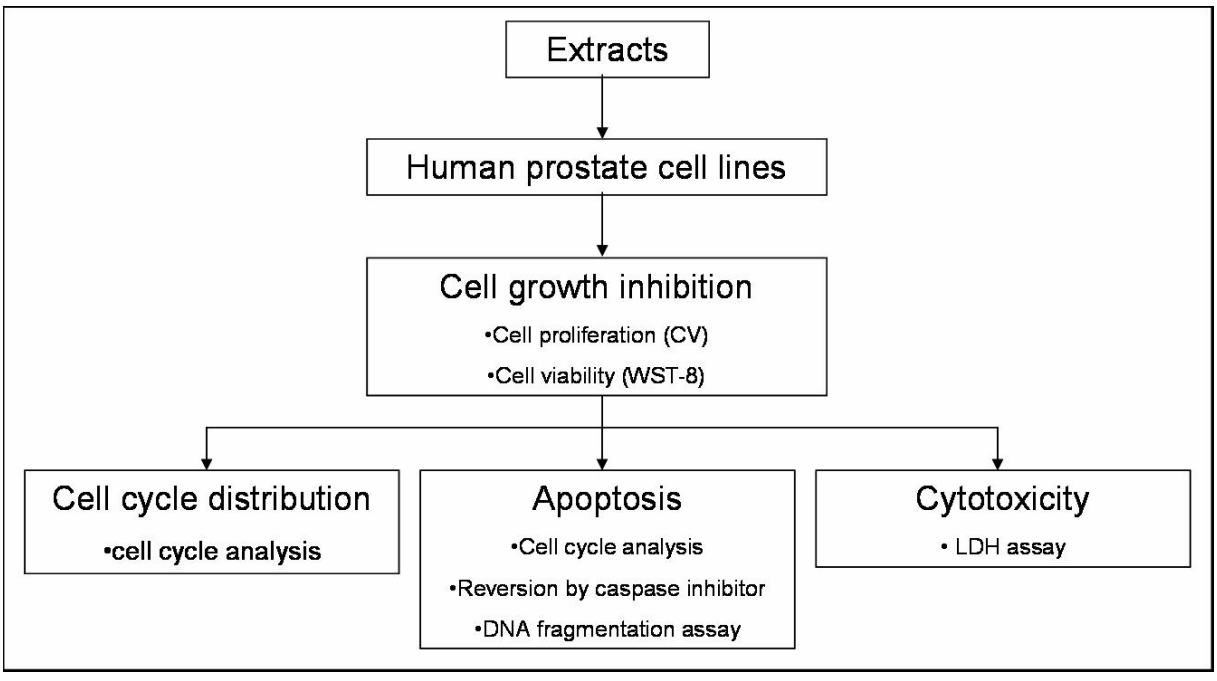


Figure III- 1 Schematic diagram of the in vitro investigations

1.2 Target cell selection

Three different cell lines, representing different disease stages, were chosen to test the effects of selected extracts on cell proliferation, induction of apoptosis, and cell cycle distribution: BPH-1, an immortalized prostate epithelial cell line established from a BPH specimen (Hayward et al 1995) and two prostate cancer cell lines that mimic the initial (hormone-sensitive) and advanced (hormone-refractory) stages of prostate carcinoma, the androgen-responsive LNCaP (Horoszewicz et al 1980), and the androgen-insensitive PC-3 (Kaighn et al 1979). Origins of the three cell lines investigated are shown in Table III-1.

Table III- 1 Prostate Cell Line Origins

Cell line	Source	First described	Reference	Characteristics	Androgen receptor protein
BPH-1	SV40 immortalised benign prostatic epithelium	1995	Hayward et al.	non-tumourigenic	absent
LNCaP	Lymph node metastasis	1980	Horoszewicz et al.	tumourigenic, metastatic	present
PC-3	Bone metastasis	1978	Kaighn et al.	tumourigenic, metastatic	absent

1.3 Quantitative measurements of cell growth inhibition

To investigate the effects on cell growth, the following assay systems were applied:

- Cell viability WST-8 tetrazolium assay
- Cell proliferation Crystal violet assay

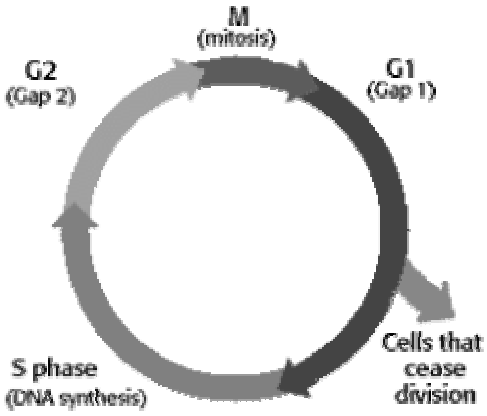
Effects of the extracts on **viability** of cultured cells were measured by a tetrazolium based WST-8 salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium], which produces a highly water-soluble formazan dye upon biochemical reduction in the presence of an electron carrier. Proliferation of cells results in an increased mitochondrial reductase system activity, which leads to increasing amounts of dark-red formazan. The amount of formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of viable cells. The soluble formazan is released into the cell culture supernatant and can be detected in situ. (Ishiyama et al 1997)

The used WST-8 reagent does not stain cells. Therefore, for measurement of **cell proliferation** additional quantitating of cells by crystal violet staining was used. Flick and Gifford (1984) introduced this technique for estimation of cytotoxicity using the basic metachromophore, crystal violet. Crystal violet (N-hexamethylpararosaniline) is a basic dye which stains cell nuclei (Gillies et al 1986). This method was modified to determine cell proliferation directly in a 96-well plate (Kueng et al 1989). The use of both assay systems was to distinguish between influences on cell proliferation and viability.

1.4 Suppression of cell division and/or induction of apoptosis

At the cellular level, reduction of cell number is controlled by a broad range of biochemical activities, either by suppressions in cell division and/or the induction of apoptosis.

Cell division is controlled by specialised checkpoints that induce the progression of cells through the cell cycle. Generally, cells do not undergo division unless they receive signals to progress in the cell cycle. Signals that direct cells to enter the cell cycle are growth factors, cytokines, or other mitogens. They induce cells to divide and trigger a large number of signal transduction cascades. Once the cell is instructed to divide, it enters the active phase of the cell cycle, which can be divided into four stages:



G1 (gap 1):	**the period of growth before DNA replication**
S (synthesis):	**as the cell undergoes DNA synthesis and replicates its genome**
G2 (gap 2):	**the cell prepared to undergo division and checks its replication using DNA repair enzymes**
M (mitosis):	**The period of cell-division**

Figure III- 2 Cell cycle phases

Following mitosis, the daughter cells may re-enter the G1 phase, or proceed to a 5th phase called G0, where growth and replication stops. Cells in G0 are said to be 'quiescent'. G0 cell may eventually re-enter G1 or perhaps die (Alberts 1994).

Stimulation of or interruption in checkpoint function results in cell cycle arrest and/or apoptosis (Hartwell & Kastan 1994). Mutations of control genes can lead to mutagenesis. The ability to interfere with this checkpoint functions is characteristic of many antiproliferating and antitumourigenic agents, including flavonoids (Knowles et al 2000) and nonsteroidal anti-inflammatory agents (Lim et al 1999; Walczak et al 2001).

Two major processes of cell death can be distinguished, apoptosis, a highly regulated process, and accidental cell death, generally defined as necrosis. (Darzynkiewicz et al 1997)

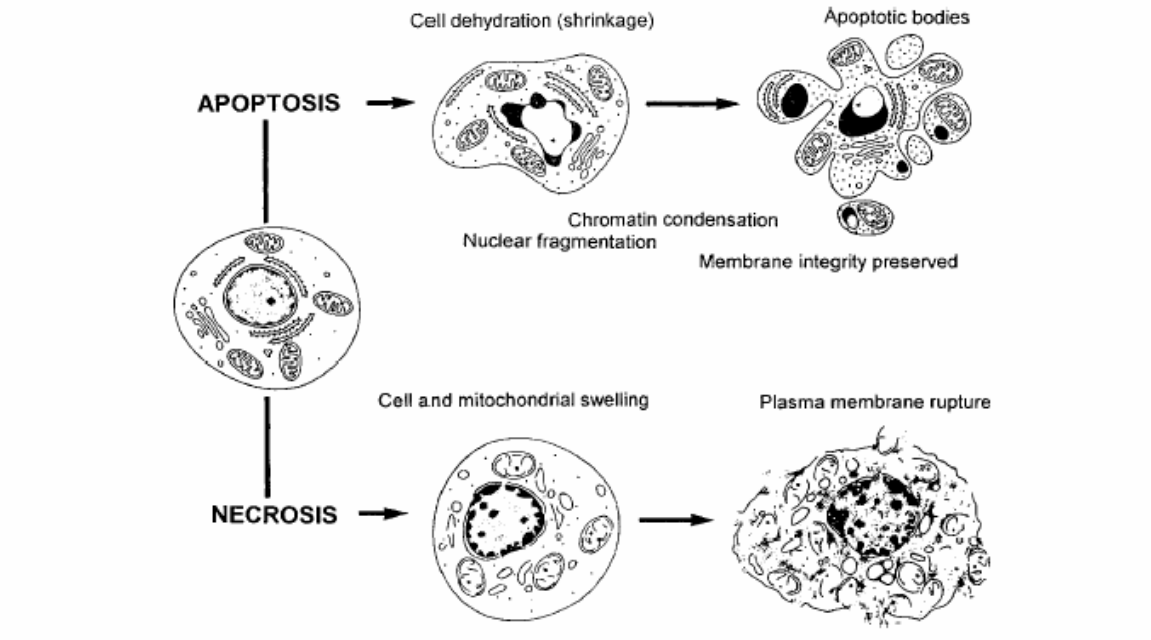


Figure III- 3 Morphological and biochemical changes during apoptosis and necrosis, reprinted from (Darzynkiewicz et al 1997). For more details see text.

In addition, certain chemical compounds and cells are said to be cytotoxic to the cell, that is, to cause its death (Table III-2)

Table III- 2 Terminology of cell death

Apoptosis:	programmed cell death, is the physiological process by which unwanted or damaged cells are eliminated during development and other normal biological processes.
Necrosis:	'accidental' cell death, is the pathological process which occurs when cell are exposed to a serious physical or chemical insult.
Cytotoxicity	is the killing property of a chemical compound or a mediator cell (cytotoxic T cell). In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism.

Apoptosis, commonly referred too as programmed cell death, is an active process which is coordinated by defined biochemical pathways. Apoptosis is the physiological mode of cell death that plays a crucial role in tissue homeostasis in multicellular organisms, both during development and in the mature organism. The deregulation of apoptosis can lead to several diseases, including cancer, autoimmune disorders and aging. Too much cell death can lead to impaired development and degenerative diseases, whereas too little cell death can lead to diseases such as cancer and persistent viral infections.(Darzynkiewicz et al 1997; Muradian & Schachtschabel 2001; Zhang & Herman 2002)

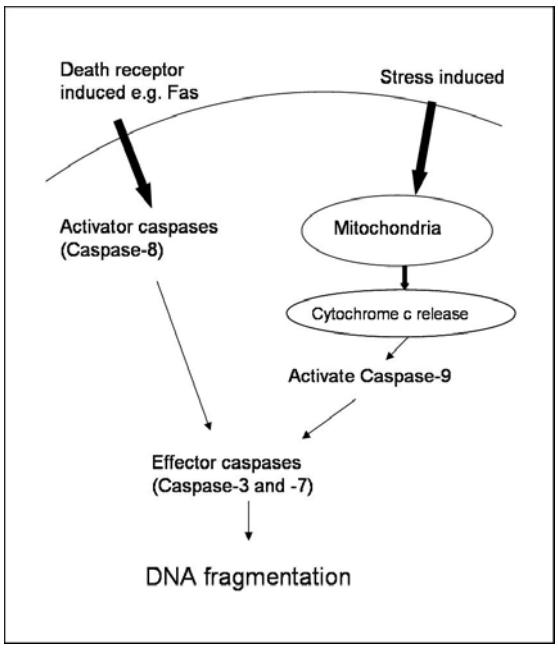


Figure III- 4 Simplified schematic diagram of extrinsic (death receptor mediated) and intrinsic (mitochondrial) pathway of apoptosis. See text for details.

Apoptosis is mediated by the activation of specific proteases, the caspases, and characterized by histological features like phosphatidylserine externalization, DNA fragmentation, nuclear condensation, and membrane blebbing. These changes occur by two major apoptotic pathways (Figure III-4), one triggered by activation of cell surface death receptors, and the other by functional inactivation of the mitochondria and their release to the cytosol of pro-apoptotic factors such as cytochrome c. The final execution phase of both pathways is mediated by activation of the caspases, a family of cysteine proteases. It has been proposed that initiator caspases with long prodomains, such as caspase-8 or -9, either directly or indirectly activate effector caspases, such as caspase-3 and -7. These effector caspases then cleave and

inactivate intracellular factors that are critical for cell survival, such as poly(ADP-ribose) polymerase (PARP) and lamins, and cause the typical apoptotic morphology described above. (Darzynkiewicz et al 1997; Granville et al 1998; Marcelli et al 1999; Zhang & Herman 2002)

When apoptosis occurs in vivo - apoptotic bodies are phagocytised by neighbouring cells, without triggering an inflammatory reaction in the tissue. In contrast, necrosis leads to swelling of the cell and finally disruption of the cell membrane. Organelles like mitochondria and nucleus remain intact throughout the process and the whole cell content is released in the surrounding tissue. Therefore the latter is often associated with inflammation in the area concerned (Granville et al 1998). Hence, induction of apoptosis in benign and malignant hyperplastic cells is the preferred way of eliminating them. In addition, an efficient apoptotic process in normal tissues prevents malignant transformation by elimination of defective cells and helps to correct age-related tissue damage (Zhang & Herman 2002).

To discriminate underlying antiproliferative mechanisms on the cellular level, several methodological approaches were performed:

- Cell cycle arrest and apoptotic ‘prepeak’ by flow cytometry
- DNA fragmentation in late apoptosis by DNA fragmentation assay
- Prevention of cell growth inhibition by adding a pan- caspase inhibitor
- Cell cytotoxicity by lactate dehydrogenase (LDH) measurement

Flow cytometry contributes significantly to **cell cycle studies** and investigations into the effects of pharmacologic agents on proliferating cells. Based on their differences in DNA content, cell cycle phases can be discriminated. Cells in G0/G1 phase have DNA content set equal to 1 unit of DNA; cells in S phase duplicate DNA, increasing its content in proportion to progression through S; and upon entering G2 and later M have twice the G0/G1 phase DNA content (2 units). The percentage of apoptotic cells can be determined from sub-G0/G1 population, indicating DNA fragmentation. The presence of a sub diploid peak is a second hallmark of apoptosis. (Darzynkiewicz et al 1997; Lim et al 1999; Darzynkiewicz et al 2001)

As shown in Figure III-4 members of the caspase family play a key role in the execution of apoptosis (Thornberry & Lazebnik 1998). Z-VAD-FMK is a cell permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases and inhibits apoptosis (Marcelli et al 1999). The peptide is O-methylated in the P1 position on aspartic acid providing enhanced stability and increased cell permeability. This inhibitor can be used as a general caspase inhibitor. Thus, the reversion by the co-treatment with Z-VAD-fmk on extract induced cell growth inhibition was investigated.

Additionally, **DNA fragmentation**, a hallmark of late apoptosis, was assessed by the specific determination of mono- and oligonucleosomes which are released into the cytoplasm of cells which die from apoptosis. The assay used is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively.

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. Thus in cell culture, the course of drug-induced **cytotoxicity** can be followed quantitatively by measuring the cytosol enzyme LDH by a tetrazolium-based kit in the cell supernatant released from damaged cells (Kumi-Diaka 2002). As it is released into the cell culture supernatant, LDH participates in a coupled reaction converting a yellow tetrazolium salt to red formazan. The amount of enzyme activity correlates to the number of damaged cells.

2 Material and Methods

2.1 Cell lines, culture conditions and cell proliferation assays

Cell cultures

Human prostate epithelial cell lines **BPH-1** (DSMZ ACC 143), **LNCaP** (ATCC CRL-1740), and **PC-3** (ATCC CRL-1435) were kindly provided by Dr. Bubendorf, Institute of Pathology, University Hospital, Basel. Cells were routinely cultured in OptiMEM 1 (Gibco BRL Life Technologies Cat. No. 31985-047) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL Life Technologies Cat.No. 10270-106) and 100μg/ml streptomycin and 100 I.U./ml penicillin (Amimed Cat. No. 4-01 F00-H) (= culture medium). Cells were grown under a humidified 5% CO_2 and 95% air atmosphere at 37°C. Medium was changed every 2-3 days. All cells used in the present study were from 10th through 30th passages.

Test extracts and compounds

Table III- 3 Extracts investigated

Plant	Extract	Extracting agent	Supplier
Serenoa repens	Fruit extract **Me180** (SabCaps®) Lot. No. C002694	Hydroethanolic (96%)	Medichemie AG (Ettingen, CH)
	Hexane green nature Fruit extract	Hexan	Vitaplant AG (Witterswil, CH)
	Hexane orange ripe Fruit extract		
	Hexane black full ripe Fruit extract		
	Ethanol green nature Fruit extract	Hydroethanolic (96%)	
	Ethanol orange ripe Fruit extract		
	Ethanol black full ripe Fruit extract		
Vitex agnus-castus	Vitex agnus-castus, FRUIT extract VACF 08	Hydroethanolic (60%)	Vitaplant AG (Witterswil, CH)
	Vitex agnus-castus, LEAF extract VAC 03-2	Hydroethanolic (60%)	Vitaplant AG (Witterswil, CH)

The extracts tested are shown in Table III-3. Me180 containing capsules (SabCaps®) were supplied by Medichemie AG, Ettingen, Switzerland. The oily extracts have been directly encapsulated into soft gelatine capsules without any additional auxiliaries, so that test samples could be taken directly out of the capsules. All others extracts were kindly provided by VitaPlant AG, Witterswil, Switzerland.

Serenoa repens plant material: fruits were collected wild in Florida at three different time points in the ripening process (unripe (=green), ripe (=orange), and full ripe (=black)). The air-dried, hackled fruits were milled and extracted with ethanol or hexane by ultrasound bath for 10 min. The solutions were filtered and evaporated under vacuum.

Solutions of the extracts were freshly prepared for each experiment by dissolution in dimethyl-sulfoxide (DMSO) and further diluted to test concentrations, before added to the experimental culture medium, giving a final concentration of 0.1% DMSO in each treatment group.

Caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK, Alexis, Cat. No. ALX-260-039) was diluted in DMSO at appropriate concentrations prior to use and added to the culture medium.

All other reagents were of the highest grade available.

2.2 Colorimetric assays for testing proliferation, viability, apoptosis and cytotoxicity

For cell growth experiments, cells were dispersed from subconfluent cultures by 0.4% trypsin in EDTA PBS (Amimed Cat. No. 5-51F) and diluted with culture medium. After determination of cell number by counting with a hemocytometer and dilution with culture medium, proliferation assays were initiated by seeding into 96-well plates (Falcon, 53 3072) at various densities in 150µl culture medium/well (BPH-1 3000 cells/well, LNCaP 1000 cells/well, PC-3 2000 cells/well) and grown for 24h at 37°C to allow cells to attach. After attachment, serum-containing medium and non-attached cells were aspirated and replaced by 100µl experimental culture medium (OptiMEM with 1% charcoal-stripped (delipidated) bovine calf serum (Sigma Cat. No.1696). Cultures were continued for another 24h before 50µl of experimental medium containing increasing concentrations of test compounds dissolved in DMSO was added and culture continued for additional time intervals (24h, 48h, 72h). Controls

received 0.1% DMSO only. In the caspase inhibitor assay, cells were co-incubated with 100µM Z-VAD-fmk.

Cell viability was quantified after indicated treatment times by adding 15µl tetrazolium-based WST-8 **Cell Counting Kit-8 Reagent** (Dojindo CK-04-13, Japan) and incubated for a further 60min at 37°C. Then the optical density (O.D.) of the dye solution was read with microplate reader (Model 3550; Bio-Rad Laboratories) at 450nm. Growth inhibition was calculated as the percent inhibition with respect to the control (control = 100%).

Cell number was determined by **crystal violet staining** as described by Kueng [8]. Briefly, if necessary after measurement of cell viability, cultures were terminated by fixing cells with 15µl of an 11% solution of glutaraldehyde (Fluka, Cat. No. 49626) (final conc. 1%) on a rotary shaker for at least 20min at room temperature. Plates were washed thoroughly with tap water and air-dried. Cells were subsequently stained with 100µl of a 0.1% solution of crystal violet (Merck, Cat. No. 1408) dissolved in 200mM HEPES Ultrol® buffer (pH 6.0) (Calbiochem, Cat. No. 391338). After being shaken for 20 minutes at room temperature, excess dye was removed by extensive washing with deionised water. The plates were air dried prior to dissolving the bound dye in 100µl 10% acetic acid. The optical density (O.D.) of the dye solution was measured at 550nm using a Bio-Rad microplate reader against acetic acid as a blank and expressed as percent of control (control =100%).

Cytotoxicity was estimated by measuring the activity of lactate dehydrogenase LDH in the cell culture supernatants using a **Cytotoxicity Detection Kit (LDH)** (Roche, No. 1 644 793). Culture medium was removed and centrifuged at 300g for 10 min to obtain a cell-free supernatant. 100µl/well of each cell-free supernatant was transferred in triplicates to wells in a 96-well microtiter plate and 100µl of LDH assay reaction mixture was added to each well. After 10 min incubation, the absorbance of the colour generated was read on a Bio-Rad microplate reader at 490 nm with a reference wavelength of 595nm. Triton-X100 (1%) was used as positive control. The mean O.D. for each treatment was normalized for cell number based on the crystal violet test group and expressed as n-fold of control (control = 1).

Apoptosis was assayed using the **Cell Death Detection ELISA plus assay** (ROCHE, No.1774 425), quantitating histone-associated DNA fragments in the cytoplasm of cells. Cells were incubated with or without the indicated concentrations

of each of the three extracts for 48h at 37°C before apoptosis was measured according to the supplier's instructions. In brief, supernatants of the cells were investigated for cytotoxicity tests (Cytotoxicity Detection Kit (LDH) see above) and attached cells were lysed in 200µL of lysis buffer per well, at room temperature for 30 min. The plates were centrifuged, and 20µL of diluted cell lysates as well as positive and negative control lysates (provided with the kit) were added to anti-histone antibody-coated assay plates. Thereafter, 80µl of the immunoreagent were added per well and the plates were incubated for 2h on a plate shaker (500 rpm at room temperature). The plates were washed three times with incubation buffer and air dried. All samples were treated with anti-DNA-peroxidase followed by development with 100µl of ABTS® substrate. The resultant absorbance (in which increasing units of absorption correlates with the increasing levels of apoptosis) was measured 5 min later with the microplate reader (405nm). The amounts of histone-DNA fragments were computed as a ratio of the control. The resulting values were normalized for cell number based on the crystal violet staining and expressed as n-fold.

2.3 Cell cycle and apoptosis analysis by FACScan

Culture and treatment of prostate cell lines were performed in 25cm^2 flasks (Falcon 35 3109) at the same density per area as in the microtiterplate assays (BPH-1 150 000 cells, LNCaP 100 000 cells, and PC-3 125 000 cells, respectively). Media were replaced after 24h by 10 ml experimental culture medium for 24h before treatment with various concentrations of *Serenoa repens* extract (10, 30, 50, 100µg/ml) or *Vitex agnus-castus* extract (3, 10, 30µg/ml). Both extracts were dissolved in DMSO and added to experimental culture medium. Experimental culture medium containing 0.1% DMSO served as the control.

After 48 h, adherent cells were harvested by trypsinization, and floating cells were recovered by centrifugation at 300g for 10min. Both adherent and floating cells were combined. The cells were washed once with phosphate buffered-saline (PBS) and fixed in ice-cold 70% ethanol.

Fixed cells were stored at 4°C until stained with propidium iodide reagent (containing 50µg/ml of propidium iodide and 100µg/ml of RNAse).The cells were incubated in the dark for 1h at 37°C and kept overnight at 4°C. Cell cycle analysis was performed using a Becton Dickinson FACScan cytometer (Becton Dickinson, San Jose, CA). For each treatment 8000 events were recorded. The DNA content of 8000 cells stained

with propidium iodide was measured with a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). Cell cycle analysis of DNA histograms was performed with the Multicycle program for Windows 3.0.

2.4 Statistical analysis

Each data set represents the mean of three independent experiments. The experiments were performed in triplicate unless otherwise stated. All statistical comparisons were made with Microsoft EXCEL, Version 7.0, using the unpaired, 2-tailed t-test, assuming unequal variance. Data are expressed as mean values ± standard deviation. Statistical significance was established at values of $p < 0.05$. Single asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. The IC 50 values were defined as the concentration which inhibited the growth of cells by 50% compared with that of control cells and were calculated by Sigma plot for Windows, Version 8.0.

3 Results

3.1 In vitro effects of *Serenoa repens*

3.1.1 Serenoa repens-induced cell growth inhibition in prostate cell lines

To determine the effects of *Serenoa repens* extracts (SRE) on cell growth and viability, human prostate epithelial cell lines (BPH-1, LNCaP, and PC-3) were incubated with increasing concentrations of different SRE ranging from 10 to 100 µg/ml. Inhibition of cell proliferation by the extracts was evaluated by crystal violet (CV) staining and inhibition of cell viability was studied with a WST-8 assay kit measuring the conversion of a tetrazolium compound to its visible formazan product by metabolically active cells.

In an initial study, the effects of exposure time were determined by treating prostate cells with a commercially available *Serenoa repens* extract Me180 for various time periods (24-72h). In all three cell lines, cell growth was inhibited in a dose- and time dependent manner. Figure III-5 contains data representing BPH-1 cells treated with Me180; however, similar results were obtained with all three cell lines. With rising treatment times, in all three cell lines, optical density (O.D.) values of stained cells by CV were steadily increasing in controls, indicating that proliferation rate of the control cells was not reduced after 72h of treatment. For further experiments, a 48h-treatment time was chosen, because initial growth inhibitory effects were detectable after 24h. Therefore, 48h ensured adequate time for studying the molecular mechanisms involved in growth inhibition.

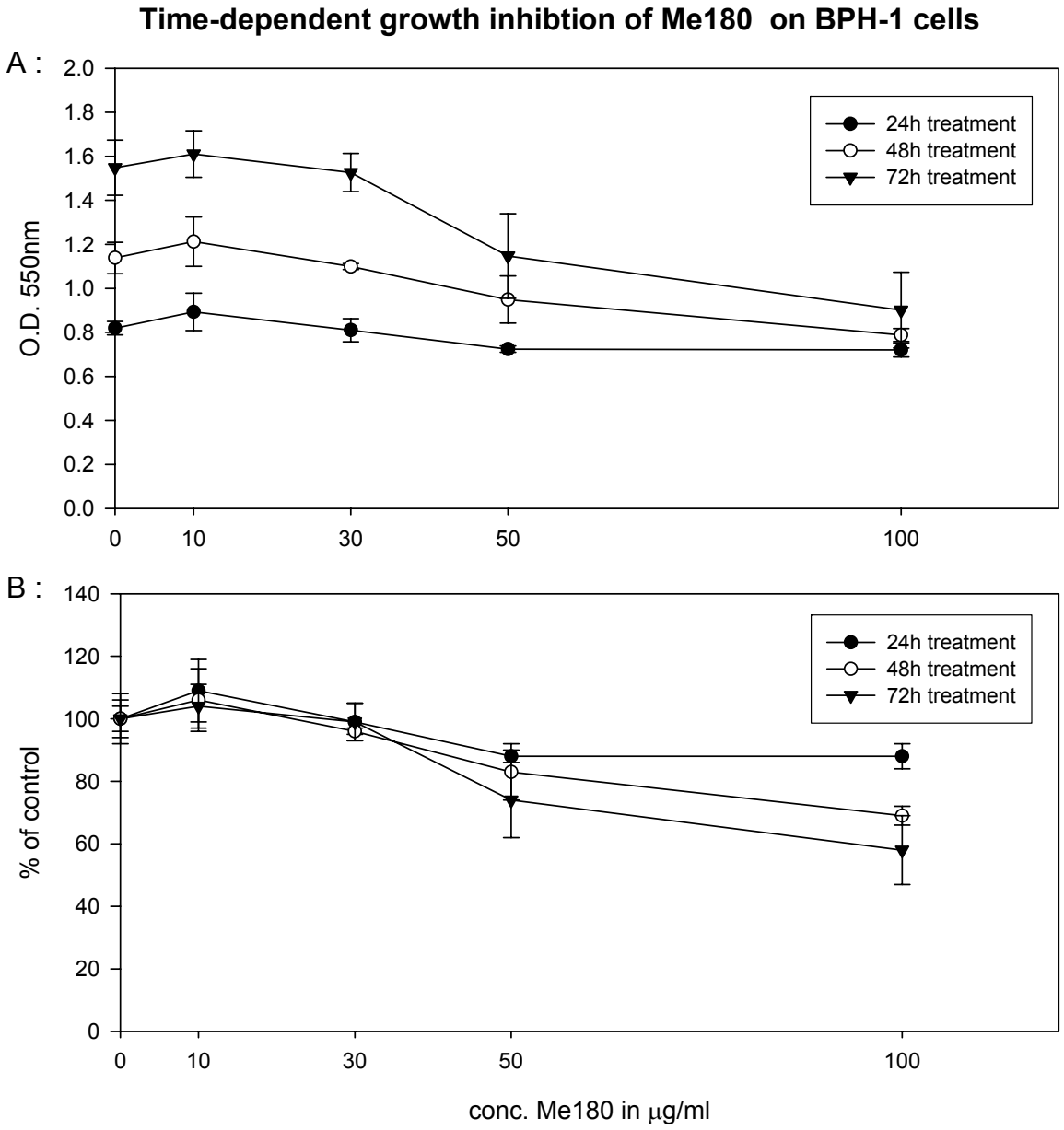


Figure III- 5 Time- and dose-dependent growth inhibition of BPH-1 cells by Me180. Cell proliferation was assessed in the presence of various concentrations of Me180. Cells were incubated for 24, 48, and 72 h. Fixed cells were stained by crystal violet staining (CV). The optical density (O.D.) of the dissolved dye was determined at 550nm. A: O.D. values are expressed as means ± SD (n = 3). B: Values are calculated as % of control (=100%) and expressed as means ± SD (n = 3).

As shown in Figure III-6, among the three cell lines, cell proliferation was more inhibited in LNCaP cells than in BPH-1 and PC-3 cells with an IC50 value of 35ug/ml. A significant suppression of LNCaP cell proliferation occurred at 30 µg/ml ($p<0.01$), showing a maximum inhibition at all concentrations from 40-100 µg/ml compared to controls. In the applied dose range, BPH-1 and PC-3 cell lines were not affected by the treatment of lower than 70µg/ml Me180 for 48h and were only significantly reduced at 100µg/ml compared to controls. Statistical analysis comparing the difference of the means at various concentrations of extract on the BPH-1 and PC-3 cell lines, showed no significance ($p>0.1$).

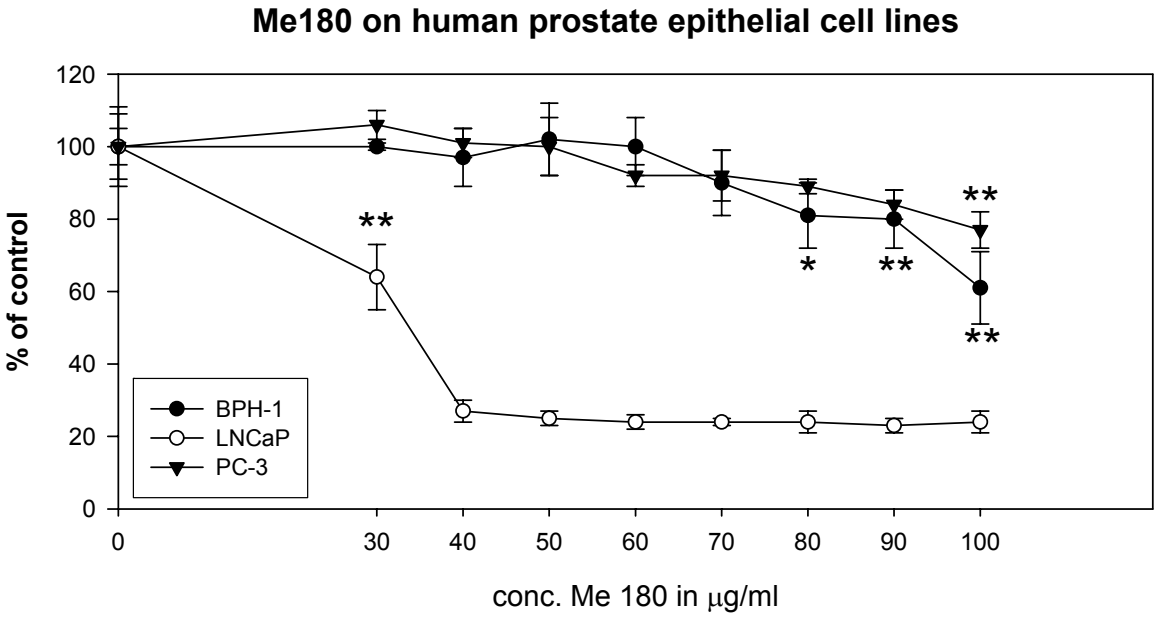


Figure III- 6 Effect of SRE Me180 on cell viability of different prostate cell lines (BPH-1, LNCaP, PC-3). Cells were incubated in the presence of various concentrations of Me180 for 48h. Cell viability was measured by WST-8. The results are expressed as % of control containing the vehicle (0.1%) in the absence of SRE. All data are means ± SD (n=3). Cell viability of LNCaP cells were significantly (, p< 0.01) inhibited after treatment of 30µg/ml of Me180 compared to solvent treated control, whereas BPH-1 cells were significantly (**, p< 0.01) inhibited after treatment of 90 and 100µg/ml of Me180 compared to vehicle treated control and PC-3 where only significantly inhibited at 100µg/ml (**, p< 0.01).**

In the WST-8 assay, the produced formazan is released into the cell medium by viable cells. Since this generated formazan dye is highly water soluble, no washing and no solubilisation steps are necessary before O.D. determination. The toxicity of WST-8 is so low that after the assay is completed, the same cells can be used for further cell proliferation measurements by the CV assay. No significant differences were found between these two assay systems, indicating that the treatment did not reduce cell viability, and thus confirm the results obtained by the two assay systems run in sequence. In an example (Figure III-7), both parameters show similar results.

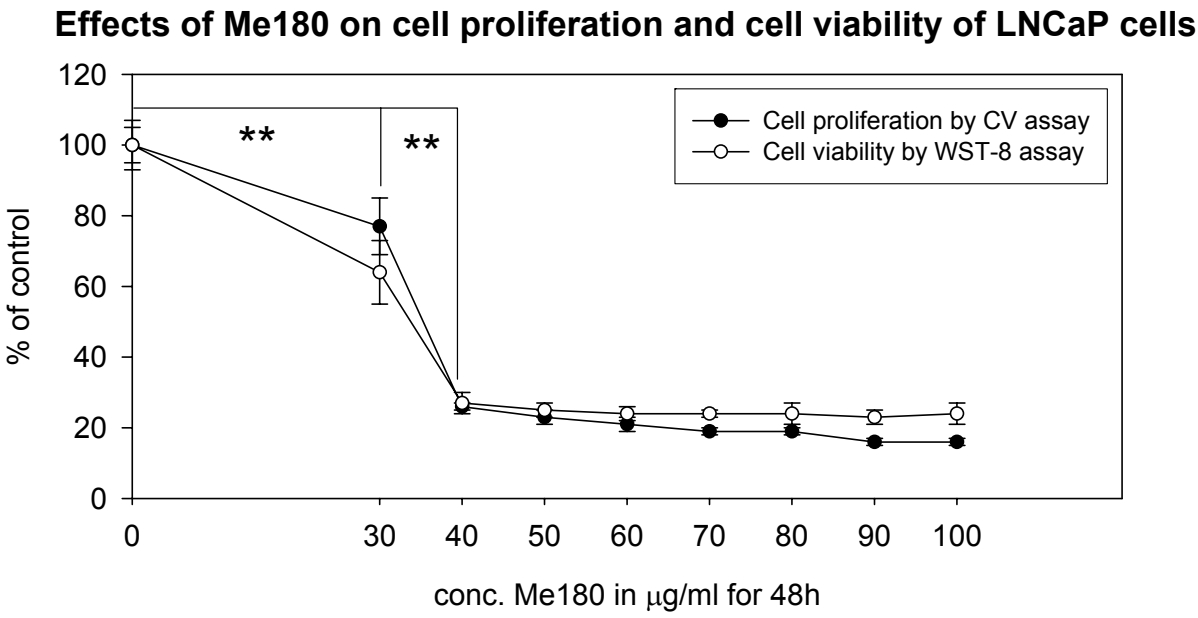


Figure III- 7 Effects of Me180 on cell proliferation and viability in LNCaP cells for 48h. No significant differences were found between the two assay systems, CV and WST-8. Cell growth was significantly inhibited by Me180 treatment in concentrations of 30-100µg/ml compared to DMSO-treated control (, p<0.01). Values are calculated as % of control (=100%) and expressed as means ± SD (n = 4).**

3.1.2 Test of different *Serenoa repens*-qualities (ripeness, extracting agent) on prostate cell growth

Serenoa repens fruits from three different ripeness stages (unripe (green), medium-ripe (orange), and full ripe (black)) were processed with 2 different extraction solvents, ethanol or hexane.

The extraction with ethanol (96%) led to a precipitate, whereas the extraction with hexane gave a homogeneous extract without precipitate. We therefore examined all different phases (mixed total extract, supernatant, and precipitate) of ethanol extracts on cell growth of the three prostate cell lines. Up to 100µg/ml no statistically significant growth inhibition was found with the precipitate in all three cell lines and by all ripeness conditions. However, the strongest growth inhibitory effect on prostate cells was found by the supernatants (Figure III-8), giving comparable effects with the commercial ethanol (96%) extract Me180 (Figure III-7).

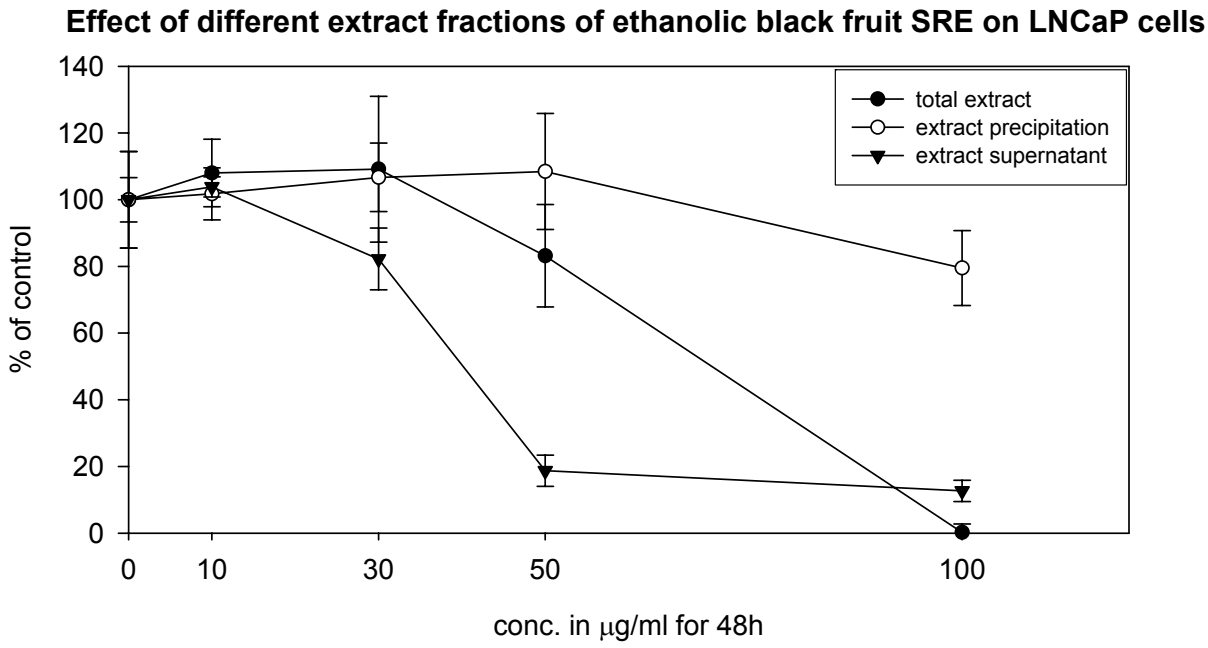


Figure III- 8 Effect of three different extract fractions (precipitation, supernatant, and total extract) of the ethanol extract of black ripe fruit of SRE on LNCaP after 48h treatment time in various concentrations. Cell proliferation was assessed by CV staining. Values are calculated as % of control (=100%) and expressed as means ± SD (n = 3). The supernatants exhibit the strongest effect, whereas cell growth was only weakly inhibited by the extract precipitations.

No differences were seen among the three ripeness conditions in the hexane extract in all three cell lines as shown in Figure III-9A. Cell lines were comparably affected as with Me180 treatment, inhibiting cell growth of BPH-1 and PC-3 only weakly at highest concentrations and exhibiting stronger effects on LNCaP cells (Figure III-7, III-9). Comparing the IC50 values, the hexane extracts exhibited significantly stronger (p= 0.03) effects, with IC50 values of 31µg/ml compared to 37µg/ml for the ethanol supernatants.

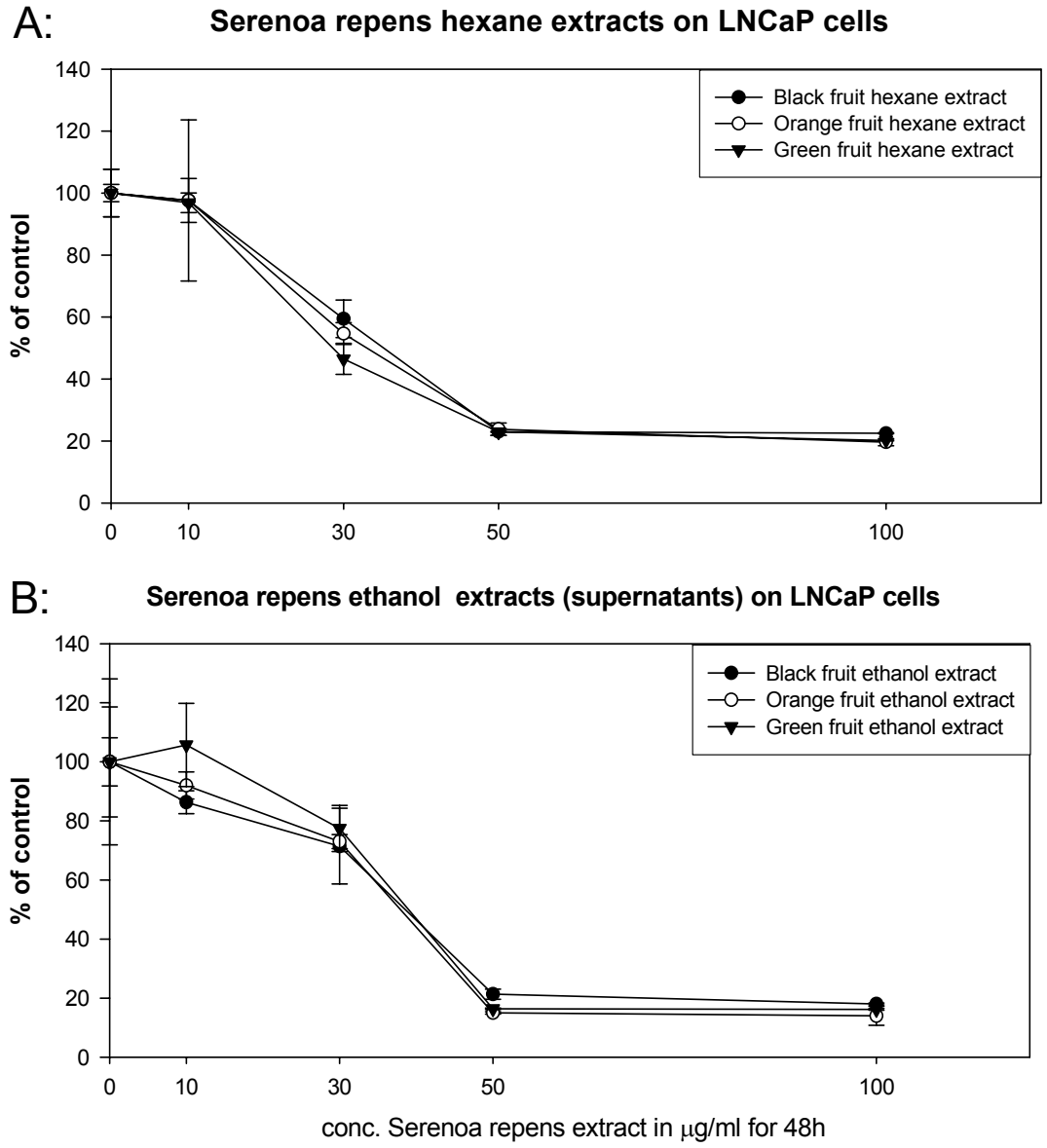


Figure III- 9 Effects of increasing concentrations of selected *Serenoa repens* extracts on LNCaP cell proliferation (CV) after 48h of treatment. Values are calculated as % of control (=100%) and expressed as means ± SD (n = 3). A: Comparison of supernatants of hexane extract of the three different ripeness conditions. B: Comparison of ethanol extracts of the three different ripeness conditions.

3.1.3 Effects of Me180 on cell cycle distribution in proliferating prostate cells

A fluorescence-activated cell sorter (FACS) method was used to test, if cell growth inhibitory effects of SRE are mediated by perturbation of cell cycle progression.

The method is based on the binding of propidium iodide to double stranded DNA. The binding is independent of base specificity and is proportional to the DNA content of a cell. It can be used to study the distribution of a given cell population in the different phases of the cell cycle and it also provides an indication of the proportion of cells undergoing apoptosis, because apoptotic cells can have a DNA content that is less than cells in G0/G1 (=pre-peak, B.A.D. background aggregates and debris). DNA content is plotted on the horizontal axis of each graph and the number of cells is plotted on the vertical axis of each graph.

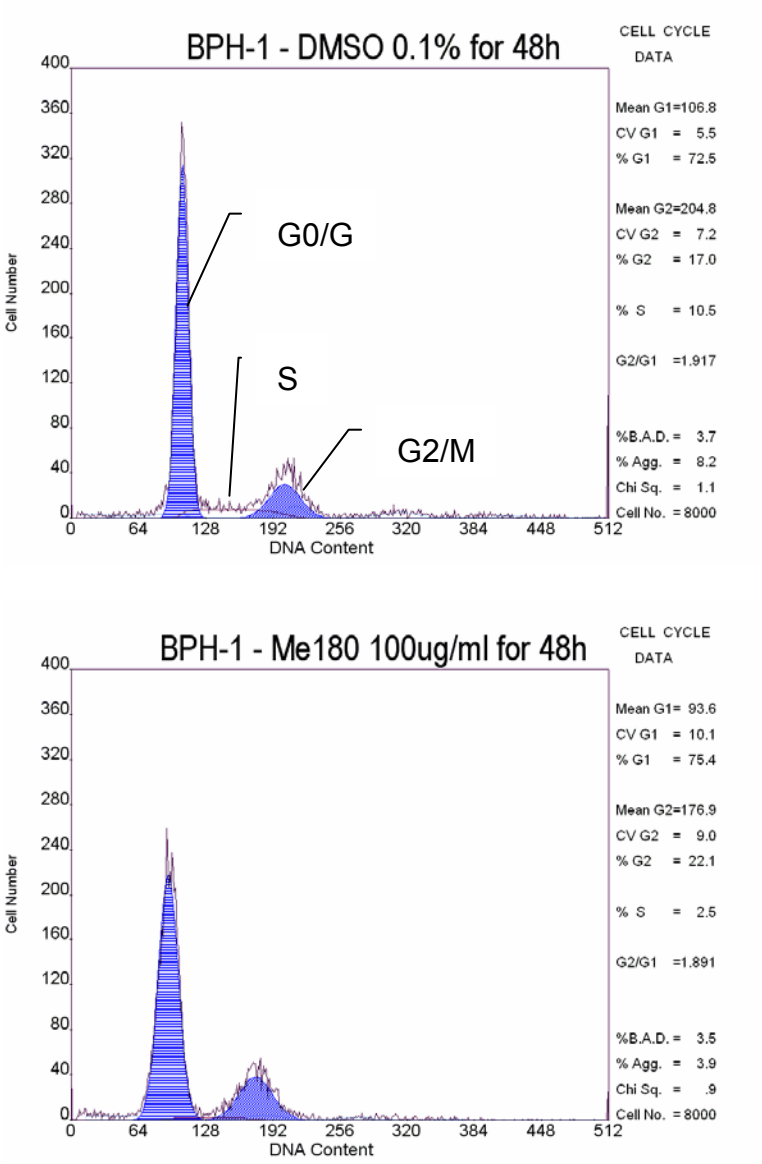
In Figures III-10, III-11, III-12, the inset depicts cartoons of the cells in the various phases of the cell cycle after treatment for 48h with *Serenoa repens* extract Me180. The percentages of cells in the different phases of the cell cycle in the DNA histograms were calculated and are shown below.

The effects appeared to be specific for each cell line. In BPH-1 cells (Figure III-10) only minor changes occurred in cycle distribution. A statistically significant ($p< 0.05$) decrease of cells in S phase (from 14.6% in controls to 7% at 100µg/ml) with a dose-dependent but non-significant increase of cells in G2/M phase was observed.

In LNCaP cells, Me180 treatment led to a statistically significant ($p<0.05$), dose-dependent increase in the number of cells in the sub G0/G1 (apoptotic) pre-peak with 1.5% in controls and 11.3% at the highest concentration of 100µg/ml, as shown in Figure III-11. Me180 treatment also resulted in an increase of the percentage of cells in the S phase. However, no more relevant shifts within the cell cycle phases were observed indicating that the extract only marginally affects LNCaP cells in cell cycle progression (Figure III-11).

In PC-3 cells (Figure III-12), treatment with Me180 exerted a dose-dependent shift from G0/G1 to S phase, with a statistically significant ($p<0.05$) decrease of the percentage of cells in G0/G1 phase from 60% to 52% and an accordant but non significant increase in the S phase from 26% to 33%.

A:



B:

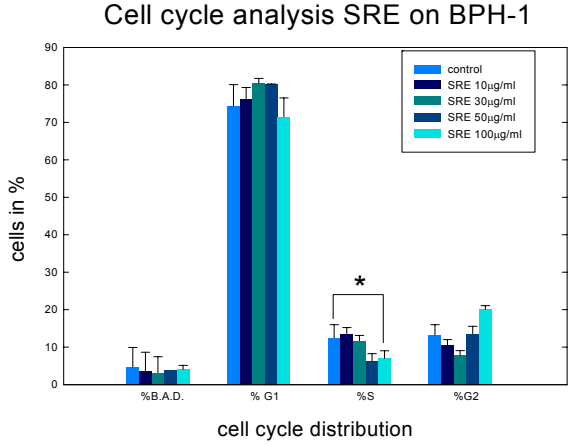
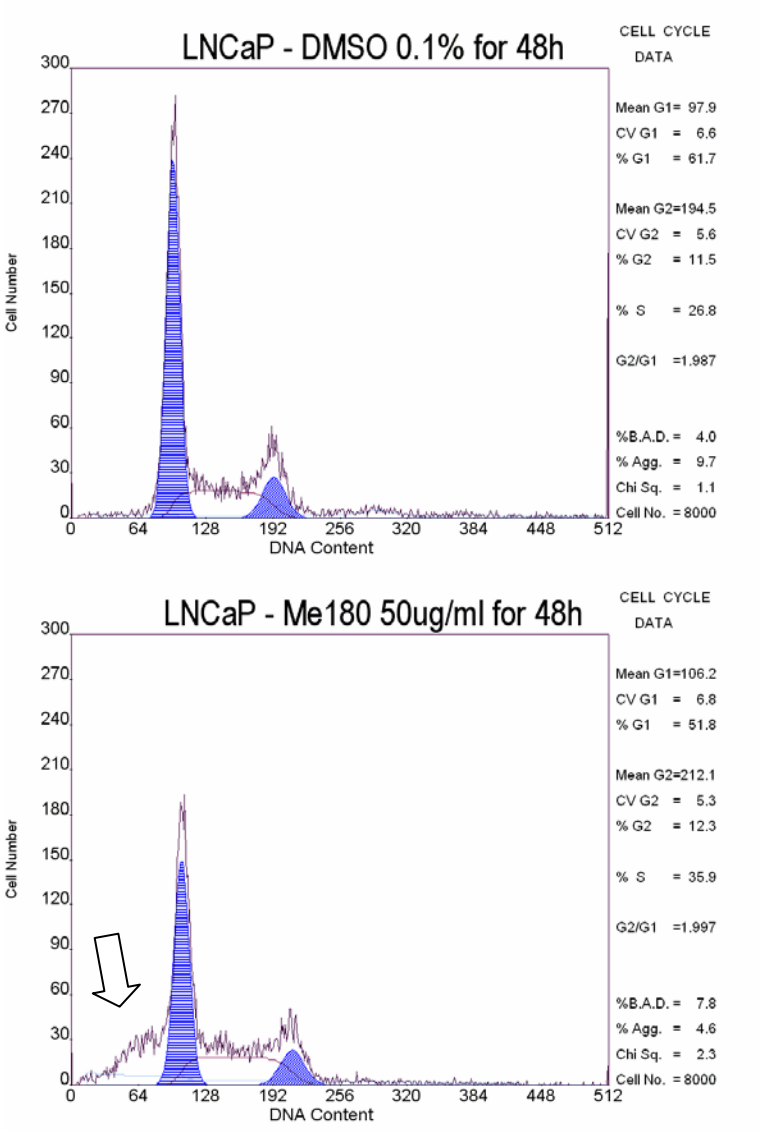


Figure III- 10 Cell cycle analysis after exposure of BPH-1 cells to Me180 for 48h.

A: Representative flow-cytometry histograms of BPH-1 control and Me180 100µg/ml B: Percentage of cells in G0/G1, S, or G2/M phase of the cell cycle and B.A.D. (apoptotic pre-peak). Values are expressed as mean ± SD from three independent experiments.

A:



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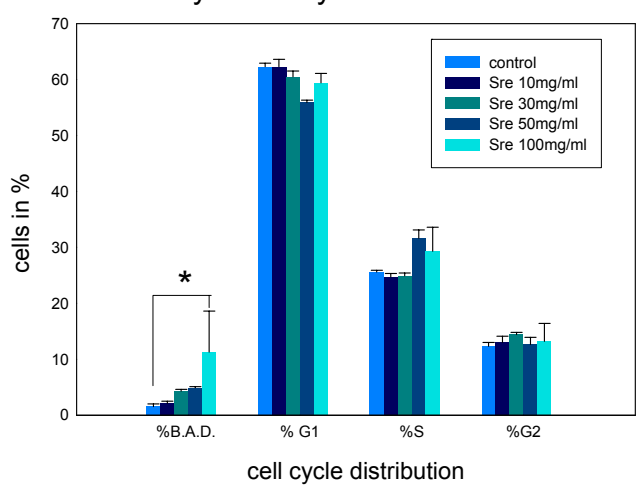
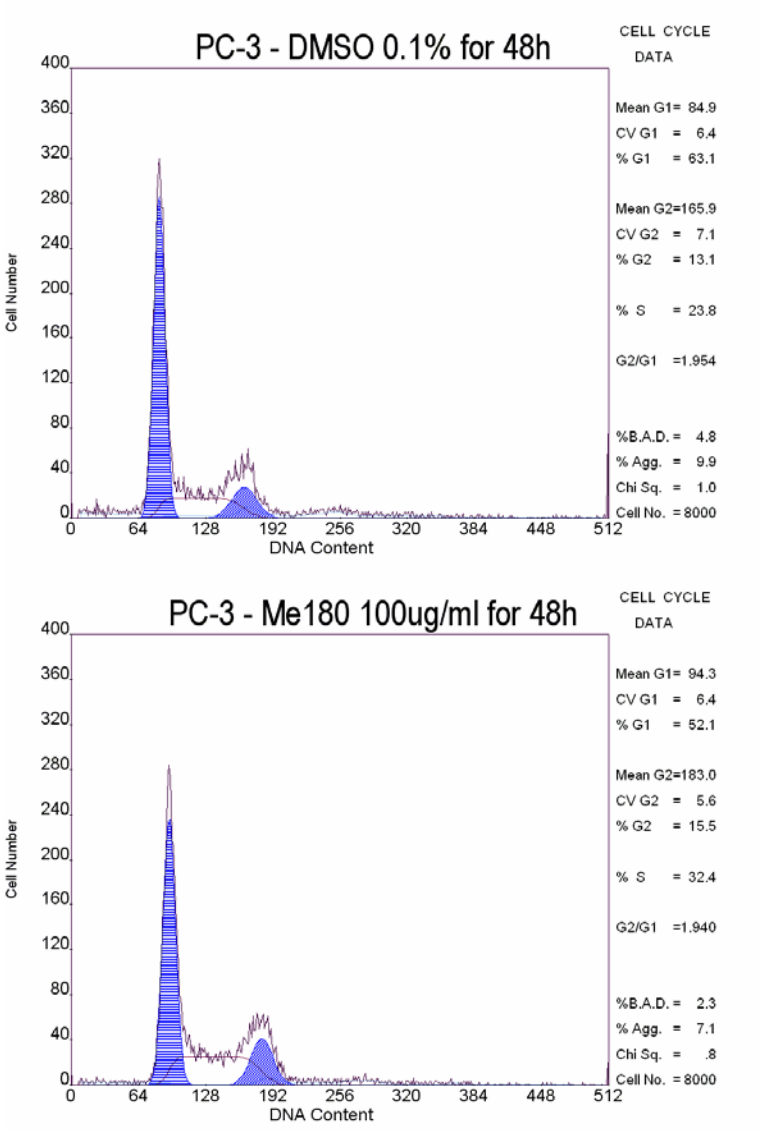


Figure III- 11 Cell cycle analysis after exposure of LNCaP cells to Me180 for 48h.

A: Representative flow-cytometry histograms of LNCaP control and Me180 50µg/ml B: Percentage of cells in G0/G1, S, or G2/M phase of the cell cycle and B.A.D. (apoptotic pre-peak, arrow). Values are expressed as mean ± SD from three independent experiments.

A:



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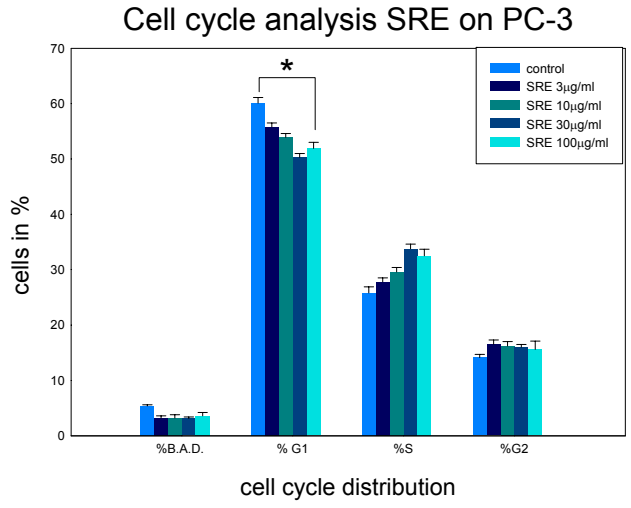


Figure III- 12 Cell cycle analysis after exposure of PC-3 cells to Me180 for 48h. A: Representative flow-cytometry histograms of PC-3 control and Me180 100µg/ml B: Percentage of cells in G0/G1, S, or G2/M phase of the cell cycle and B.A.D. Values are expressed as mean ± SD from three independent experiments.

3.1.4 Induction of apoptosis and cytotoxic effects by *Serenoa repens* extract

Cell cycle studies demonstrated that in all three cell lines only minor effects occurred in cell cycle progression after Me180 treatment. To clarify this effect observed with respect of apoptosis and cell cytotoxicity, we further applied two different assay systems, one to assess the reversion of apoptosis by a pan-caspase-inhibitor (Z-VAD-fmk) interfering with the apoptotic process, and the other, to assess the induction of DNA fragmentation, a hallmark of late apoptosis.

As shown in Figure III-13, cell growth inhibition in BPH-1 cells was only weakly reversed by co-treatment with the caspase inhibitor. As expected, the effect was stronger in LNCaP cells as compared to BPH-1 cells. In cell cycle analysis Me180-treated LNCaP cells revealed a G0/G1 “pre-peak” indicating that apoptotic cell death might be involved (Figure III-11). The reversion of Me180-induced cell growth inhibition by co-treatment with the pan-caspase inhibitor Z-VAD-fmk (100µM) demonstrated that this was due in part to apoptosis (Figure III-13). However, this reversion was only statistically significant at low concentrations of 25µg/ml of extract ($p<0.05$). No reversion was found in PC-3 cells.

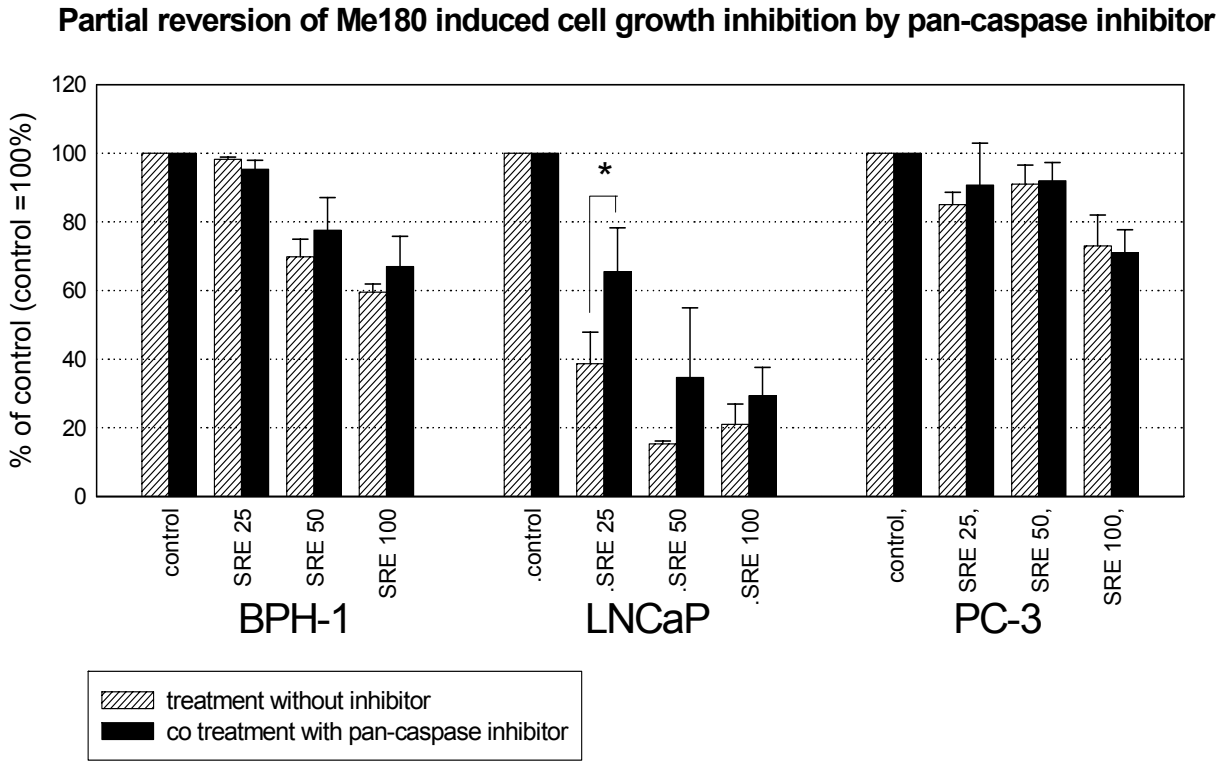


Figure III- 13 Me180-induced cell growth inhibition is partially reversed by co-treatment with the pan-caspase inhibitor Z-VAD-fmk 100µM for 48h. Fixed cells were stained by crystal violet. Optical density of the dissolved dye was determined at 550nm and calculated as % of control (=100%) and expressed as means ± SD of three independent experiments. Reversion of cell growth inhibiting effects was statistically significant only in LNCaP cells at 25µg/ml extract (*, $p<0.05$).

Based on observed effects, DNA fragmentation, an event of late apoptosis, was evaluated as a further indicator of apoptosis after treatment of prostate epithelial cells with Me180. Furthermore, lactate dehydrogenase (LDH) in the cell supernatant was assessed to evaluate, whether Me180-induced cell growth inhibition was also partly due to cytotoxicity.

Concentrations of 50µg/ml and 100µg/ml Me180 were tested for 48h in all three prostate cell lines. As shown in Figure III-14, our results suggest two different modes of cell death involved: through an apoptotic pathway leading to enhanced DNA fragmentation after the treatment with Me180 and secondly via a cytotoxic effect leading to an increase of LDH activity in the cell supernatants of treated cells compared to untreated cells.

BPH-1 cells treated with Me180 displayed a statistical significantly ($p<0.01$) dose-dependent increase of DNA fragmentation (2.6-fold for 50µg/ml and 3.8-fold for 100µg/ml compared to untreated controls), corresponding to previous results obtained by reversion with the caspase inhibitor (Figure III-13), but in contrast to the cell cycle analysis (Figure III-10), were no apoptotic pre-peak was observed.

Moreover, corresponding to cell cycle analysis (Figure III-11) and reversion by co-treatment with the caspase inhibitor (Figure III-13), LNCaP revealed also a dose-dependent increase in DNA fragmentation with 3-fold with 50µg/ml and 4.3 fold with 100µg/ml SRE, respectively, showing further evidence of apoptosis.

In contrast, in PC-3 cells Me180-treatment did not result in apoptosis as evidenced by a lack of DNA fragmentation (Figure III-14). These observations were consistent with the lack of the apoptotic pre-peak in cell cycle analysis (Figure III-12) and the lack of a reversion of cell growth inhibition by the caspase inhibitor (Figure III-13). The PC-3 cells thus appear to be insensitive to Me180-treatment.

Consistent with these findings, LDH leakage, as a marker of necrosis, increased more in LNCaP cells than in PC-3 and in BPH cells. Higher concentration of 100µg/ml of SRE caused statistically significant increase of LDH in the cell supernatant ($p<0.01$), indicating more severe cellular damage in LNCaP, whereas in BPH-1 and PC-3 cells no change in LDH was detected as compared to controls (Figure III-14).

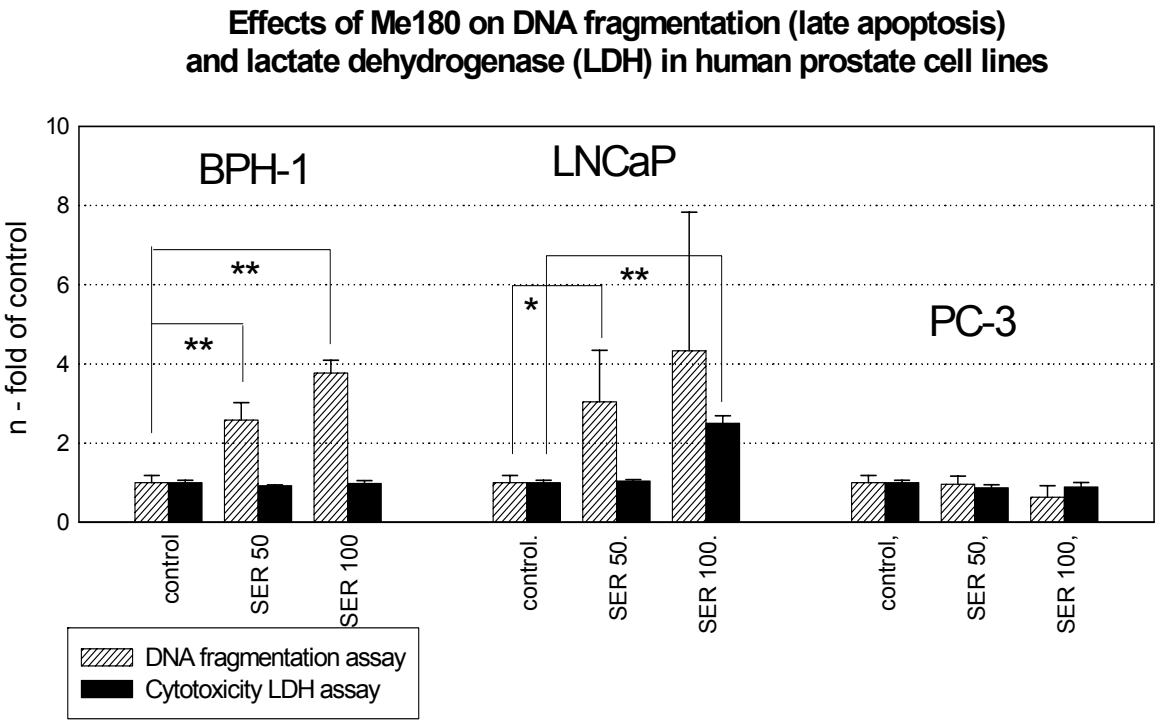


Figure III- 14 Induction of DNA fragmentation and LDH leakage after exposure of prostate cell lines to *Serenoa repens* extract Me180 for 48h. Values are calculated as n-fold of control (n=1) and expressed as means ± SD of three independent experiments. Dose-dependent statistically significant (, p<0.01) increase of DNA fragmentation was only observed in BPH-1 cells, whereas a significant (*, p<0.05) increase was also observed in LNCaP cells with 50µg/ml of extract. Statistically significant increase of LDH in the cell supernatant was only found in LNCaP at the highest concentration of 100µg/ml (**, p<0.01).**

3.2 Effects of *Vitex agnus-castus* extracts in prostate cell lines

3.2.1 Antiproliferative response of cells to extracts of *Vitex agnus-castus*

Recently, a *Vitex agnus-castus* fruit extract (VACF) was shown to exhibit anti-tumour activities in different human cancer cell lines (Ohyama et al 2003), but its effects on human prostate epithelial cells has so far not been investigated. As for SRE-treatment, the effects of VACF were first determined by incubating prostate cells with various doses ranging from 1 to 30µg/ml, and 3 to 100µg/ml of VACF and *Vitex agnus-castus* leaf extract (VACL), respectively, for various time periods (24-72h). Figure III-15 illustrates the dose- and time-dependent effects in BPH-1 cells by VACF, as a representative example.

Cell proliferation and viability was equally affected in all three human prostate cell lines (BPH-1, LNCaP, and PC-3) by a significant decrease after VACF-treatment compared to controls. The difference between treated and untreated cells was already significant ($p<0.05$) after 24h and this difference became more prominent and profound over time. Inhibition of cell growth was significant ($p<0.01$) at 10 and 30 µg/ml for all three cell lines after treatment of 48h (Figure III-17).

Comparing fruit and leaf extracts, VACF (fruit) was the more potent extract, causing significant inhibition of cell growth with IC50 values of 7, 8 and 7µg/ml in BPH-1, LNCaP, and PC3, respectively. VACL (leaf) treatment also inhibited prostate cell growth, but at approximately two-fold higher concentrations, corresponding to IC50 values of 17, 15 and 20 µg/ml (Figure III-16).

Similarly to the results with SRE-treatment, comparable data were obtained with WST-8 assay and with CV assay, respectively, showing that the amount of the formazan produced by viable cells corresponds to the number of attached cells stained by CV. A 48h growth inhibition curve of each cell line after treatment with VACF comparing the two methods is depicted in Figure III-17.

The next series of experiments were performed to gain further information on the mechanism of *Vitex agnus-castus* mediated growth inhibition.

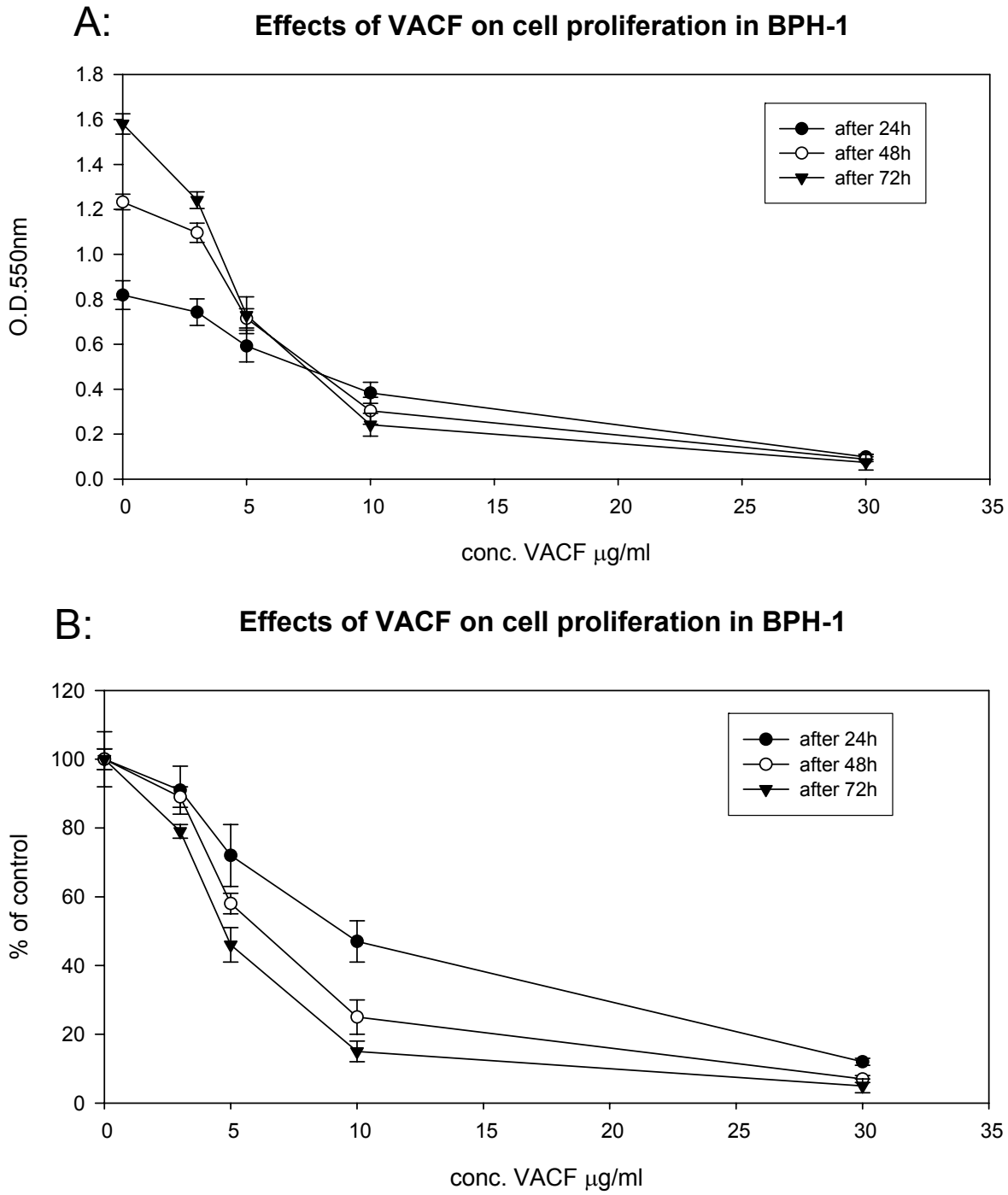


Figure III- 15 Time- and dose-dependent growth inhibition of proliferation of BPH-1 cells by Vitex agnus-castus fruit extract (VACF). Cells were incubated with various concentrations of VACF for various time periods (24, 48, and 72 h). Fixed cells were stained by crystal violet. The optical density (O.D.) of the dissolved dye was determined at 550nm. A: Optical density (O.D.) values are expressed as means ± SD (*n* = 3). B: Values are calculated as % of control (=100%) and expressed as means ± SD (*n* = 3).

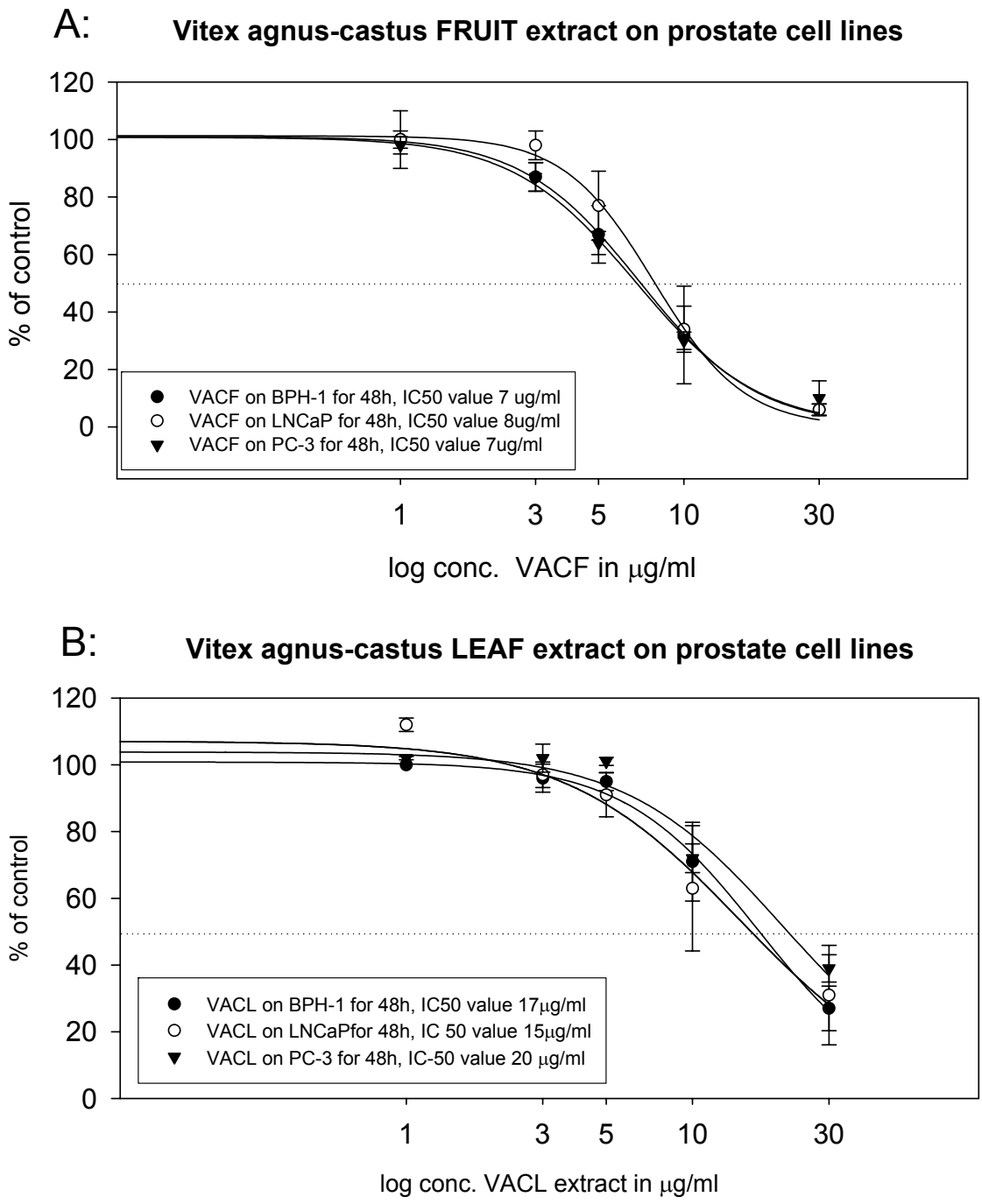


Figure III- 16 Growth inhibition of prostate cell lines by VAC- treatment for 48h. Fixed cells were stained by crystal violet staining. The optical density (O.D.) of the dissolved dye was determined at 550nm. Values are calculated as % of control (=100%) and expressed as means ± SD (*n* = 3). A: *Vitex agnus-castus* fruit extract, B: *Vitex agnus-castus* leaf extract.

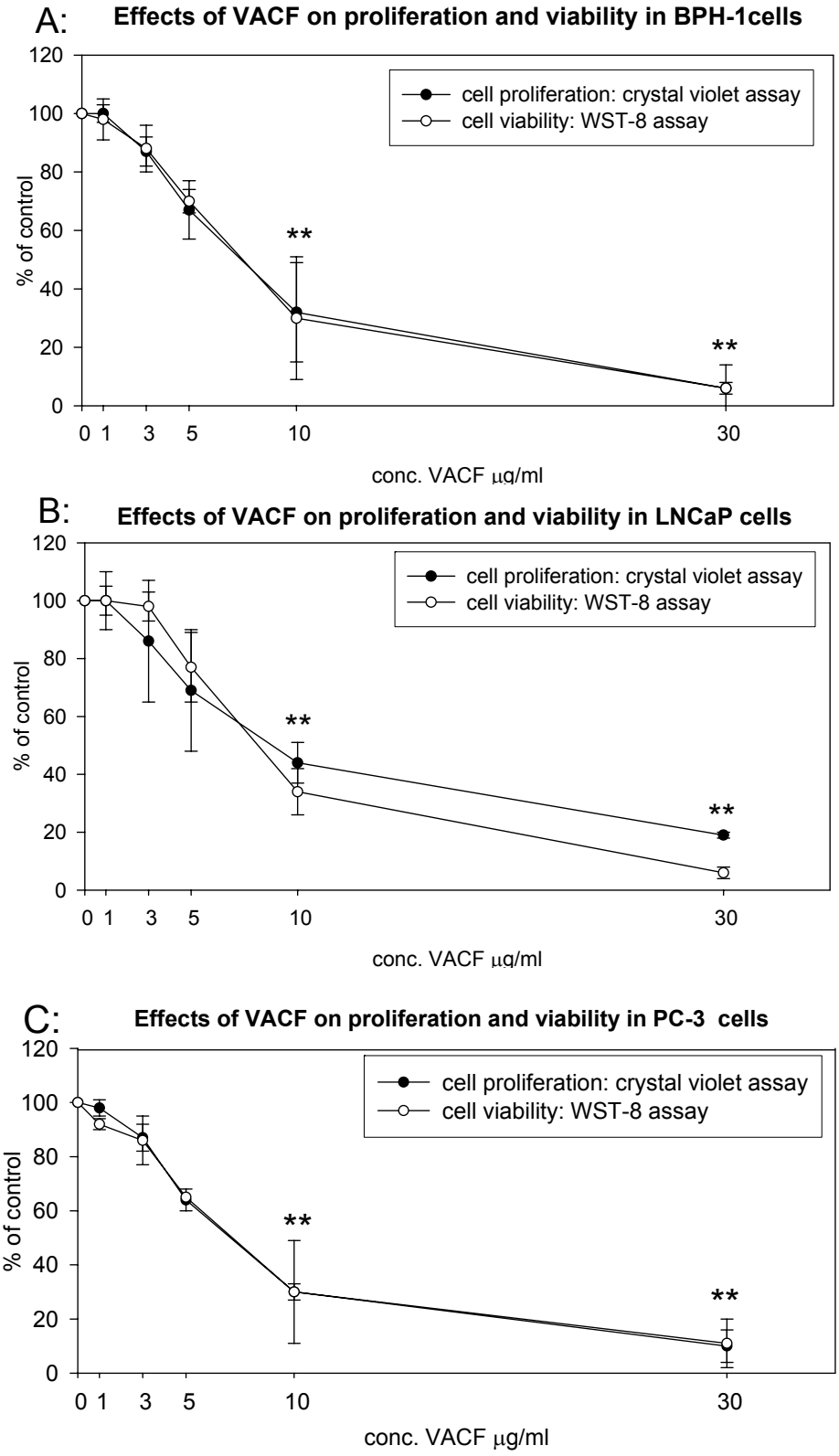


Figure III- 17 Effects of VACF on cell proliferation and viability in prostate cell lines cells for 48h. No significant differences were assessed between the two assay systems, CV and WST-8. Cell growth was significantly inhibited by VACF at 10 and 30 µg/ml in all three cell lines compared to untreated controls (, p<0.01). Values are calculated as % of control (=100%) and expressed as means ± SD (*n* = 4).**

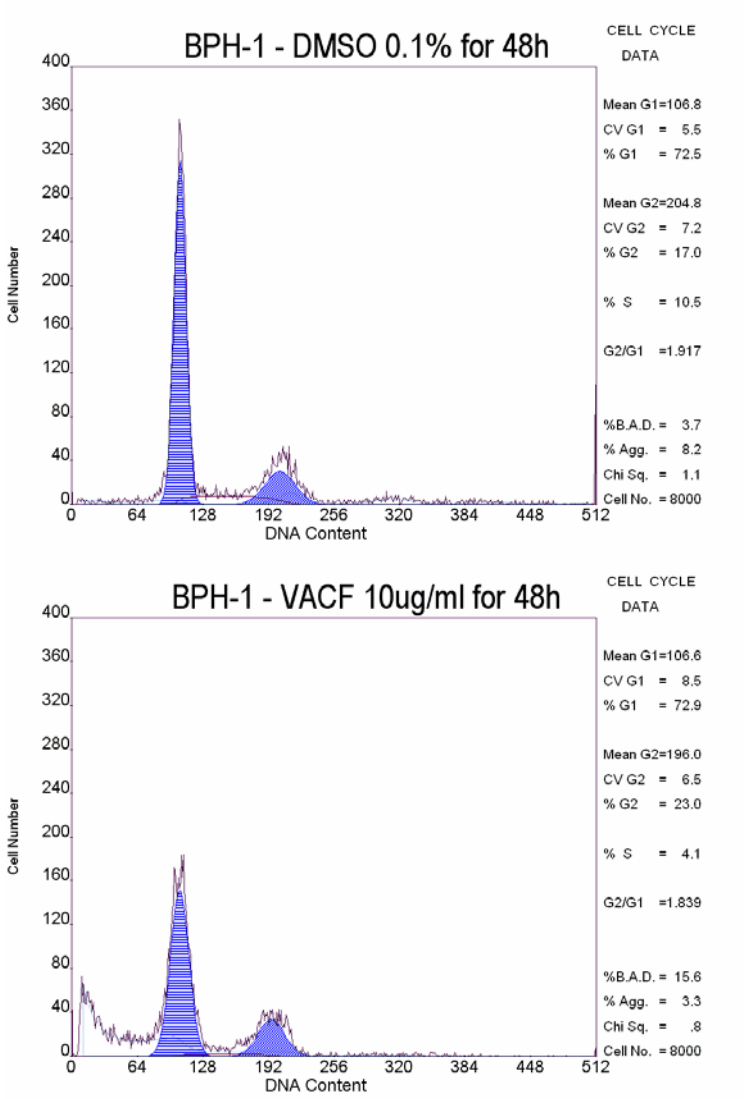
3.2.2 Effects of VACF on cell cycle distribution in proliferating prostate cells

Cell proliferation assays indicated that VACF significantly reduced the survival of human prostate cell lines, leading us to further investigate the molecular mechanisms of cell death involved. To determine whether the inhibition of cell growth was due to apoptosis and/or cell cycle arrest, cell cycle analysis wascarried out by flow cytometry for all cell lines investigated. As mentioned before, propidium iodide staining of cellular DNA, followed by FACS analysis, provides a useful method for evaluating the cell cycle. In Figures III-18-20 we present cell cycle distribution data after treatment with 10µg/ml VACF for 48h. Percentages of cells in the different phases of the cell cycle after exposure to VACF in various concentrations (3,10,30 µg/ml) for 48h are displayed in the figures below.

VACF treatment clearly altered the pattern of cell cycle distribution in all three prostate cell lines: The flow cytometric analysis showed, that solvent treated controls of all three cell lines gave little or no events recorded in the sub G0/G1 (apoptotic) pre-peak and displayed a distribution of cells in the G0/G1, S and G2/M phases typical of proliferating cancer cell lines. The number of cells in the sub G0/G1 pre-peak increased dose-dependently after 48h of treatment in all three cell lines. In a concentration of 30µg/ml VACF, this increase was statistically significant in all three cell lines ($p<0.01$). As mentioned earlier, this additional sub G0/G1 pre-peak is characteristic for apoptotic cells and represents a first indicator for DNA fragmentation.

Additionally, in BPH-1 cells treatment with VACF resulted in a dose-dependent decrease of cell number in G0/G1 phase compared to untreated controls being significant at 30µg/ml ($p<0.05$), and displayed a concomitant increase in the percentage of cells in G2/M phase, statistically significant at 30µg/ml ($p<0.01$) shown in Figure III-18. These effects were less clear after treatment of LNCaP cells (Figure III-19). In PC-3 cells, the treatment with VACF revealed a dose-dependent, but non-significant decrease of the percentage of cells in G0/G1 phase with a corresponding dose-dependent but significant ($p<0.05$) increase of cells in the S phase (Figure III-20).

All data showed treatments of 48h of VACF exposure. Similar effects by VACF were observed after prolonging treatment time to 72h.



Cell cycle analysis VACF on BPH-1

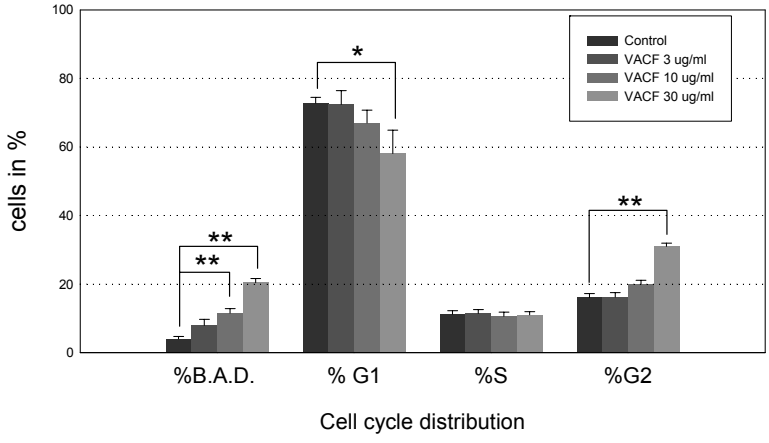
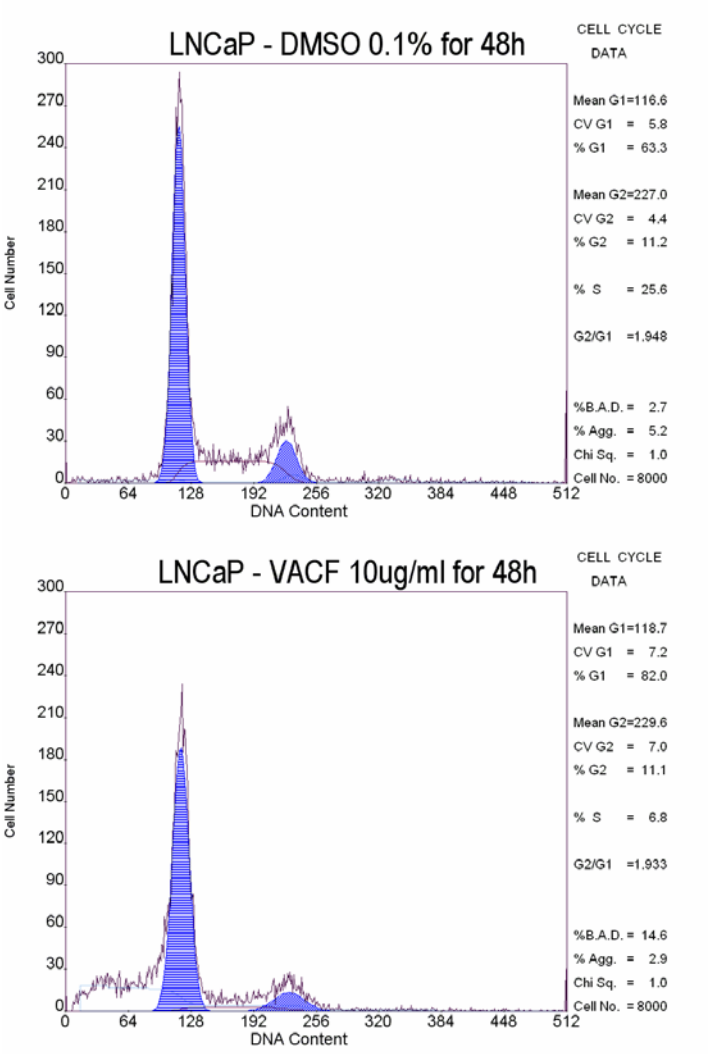


Figure III- 18 Cell cycle analysis: BPH-1 cells were exposed to various concentrations of VACF for 48h. Histograms show cell cycle distribution of cells treated with or without VACF 10µg/ml for 48h. The percentage of cells in each phase was calculated based on the DNA histogram and are displayed for increasing concentration of VACF in the figure below. The values represent the means ± SD of three independent experiments.



Cell cycle analysis VACF on LNCaP

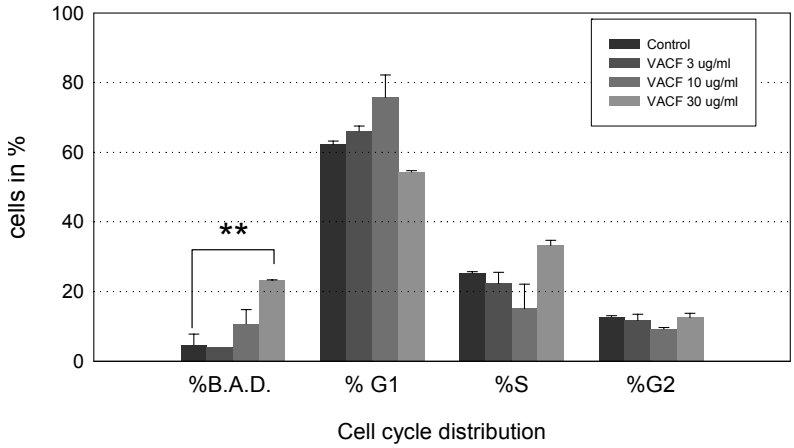
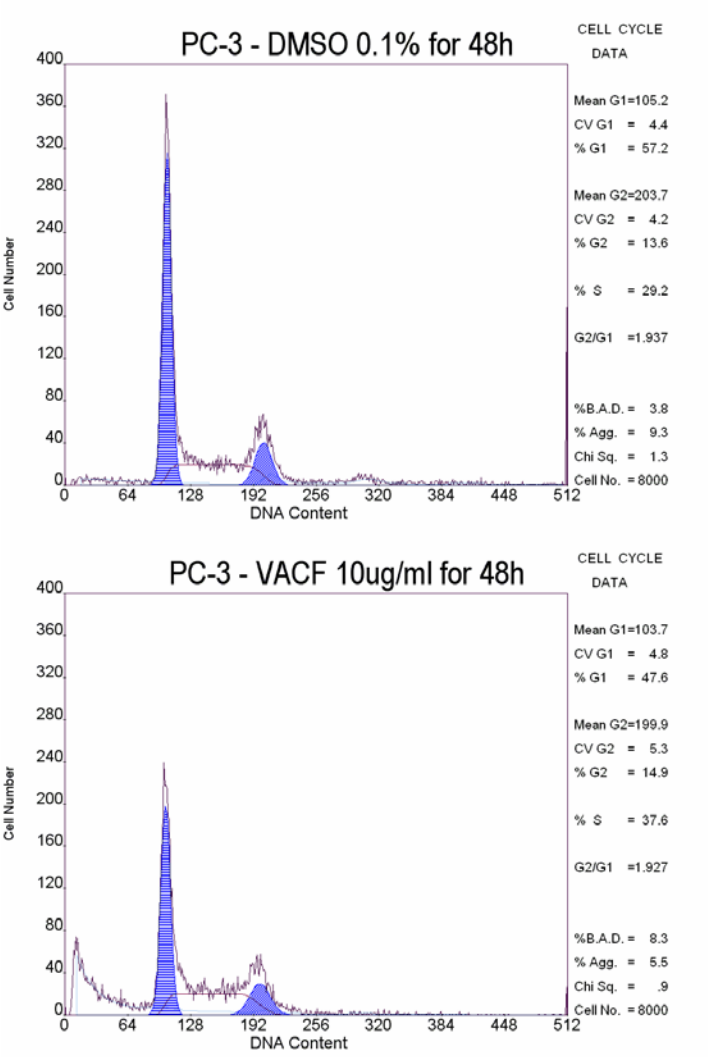


Figure III- 19 Cell cycle analysis: LNCaP cells were exposed to various concentrations of VACF for 48h. Histograms show cell cycle distribution of cells treated with or without VACF 10µg/ml for 48h. The percentage of cells in each phase was calculated based on the DNA histogram and are displayed for increasing concentration of VACF in the figure below. The values represent the means ± SD of three independent experiments.



Cell cycle analysis VACF on PC-3

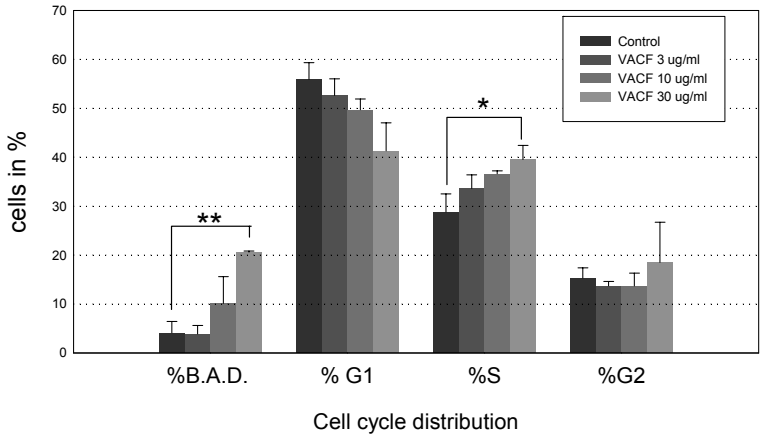


Figure III- 20 Cell cycle analysis: PC-3 cells were exposed to various concentrations of VACF for 48h. Histograms show cell cycle distribution of cells treated with or without VACF 10µg/ml for 48h. The percentage of cells in each phase was calculated based on the DNA histogram and are displayed for increasing concentration of VACF in the figure below. The values represent the means ± SD of three independent experiments.

3.2.3 Estimation of induction of apoptosis and cytotoxicity by VACF

Further assays were performed to investigate the effects of VACF- and VACL - induced cell death. Most of the morphological changes that are observed during the apoptotic process are caused by a set of cysteine proteases in the caspase cascade, which are activated especially in apoptotic cells. Therefore, prostate cell lines were exposed to VAC together with a broad spectrum caspase inhibitor. As shown in Figure III-21 and III-22, the caspase inhibitor Z-VAD-fmk (100µM) reversed the VAC growth inhibiting effect in all three cell lines investigated. The modest reversion of VACF and VACL induced cell growth inhibition by co-treatment with the pan-caspase inhibitor Z-VAD-fmk demonstrated that cell growth inhibition is partially due to caspase-mediated apoptosis. However, statistical significance for reversion of VACF-induced cell growth inhibition was only achieved in BPH-1 cells at concentrations of 10 and 30 µg/ml and in LNCaP cells at 30µg/ml ($p<0.05$), and for VACL-induced cell growth inhibition at 30µg/ml in BPH-1 ($p<0.01$) and in PC-3 cells ($p<0.05$).

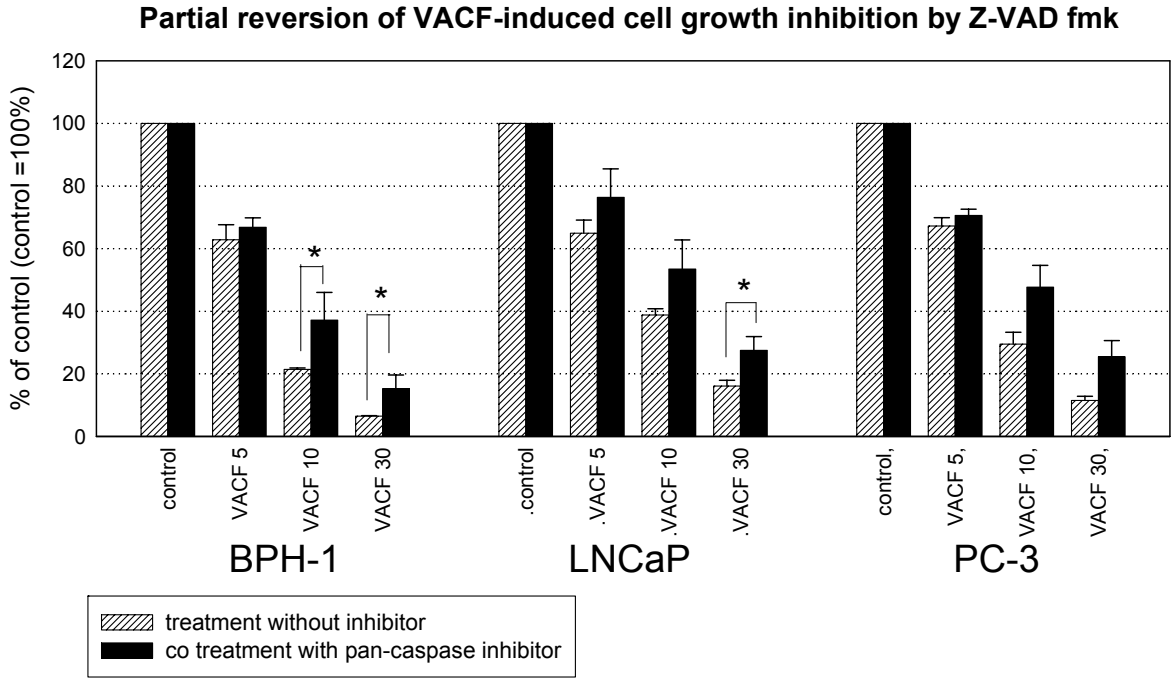


Figure III- 21 VACF-induced cell growth inhibition is partially reversed by co-treatment with pan- caspase inhibitor Z-VAD-fmk (100µM) for 48h. Fixed cells were stained by CV. O.D. of the dissolved dye was determined at 550nm and calculated as % of control (=100%) and expressed as means ± SD (n = 3). (* = $p<0.05$)

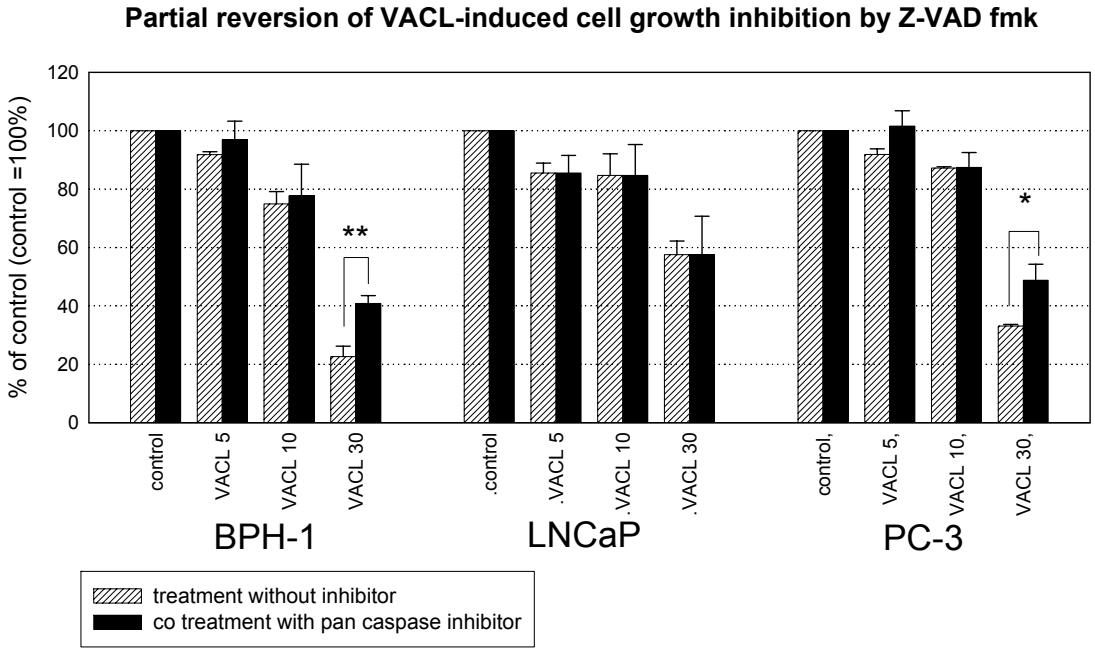


Figure III- 22 VACL-induced cell growth inhibition is partially reversed by co-treatment with pan- caspase inhibitor Z-VAD-fmk 100µM for 48h. Fixed cells were stained by CV. O.D. of the dissolved dye was determined at 550nm and calculated as % of control (=100%) and expressed as means ± SD (n = 3) (*,p<0.05 and **, p<0.01).

To further investigate the apoptotic effects and to differentiate between apoptosis and cytotoxicity, we further used a combined assay system detecting DNA fragmentation in the cells (ELISA) and LDH in the cell supernatants of the same cells. The data obtained show that VACF and VACL reduce human prostate cell proliferation in culture partly by apoptosis. However, in addition, the increased LDH leakage indicated that inhibition of proliferation was also partly due to a cytotoxic reaction (Figures III-23 and III-24).

A dose-dependent increase of VACF-induced DNA fragmentation, a hallmark of late apoptosis, was found in all three cell lines. In BPH-1 cells fruit and leaf extract exhibited a statistically significant enhancement at concentrations of 10 and 30 µg/ml by fruit ($p<0.01$) and leaf extracts ($p<0.05$). In LNCaP and PC-3 cells, the apoptosis-inducing activity of both extracts was dose-dependent, being observed at concentrations as low as 5µg/ml subsequently increasing with higher concentrations of 10µg/ml.

A similar pattern of cell death was observed by LDH measurement in the cell supernatant, as an indicator of cell membrane damage with subsequent release of cytosolic LDH. VACF showed a dose-dependent and significant increase of LDH in

the cell supernatant of all three cell lines. However, in all three cell lines apoptosis reaction was higher than cytotoxic effects compared to controls. Comparable results were obtained by investigating VACL (Figure III-24). Released LDH was only significantly enhanced in BPH-1 and LNCaP at the highest concentration of 30µg/ml VACL ($p < 0.01$) whereas no increase was shown in PC-3 cells.

Effects of VACF on DNA fragmentation and LDH release in human prostate cell lines

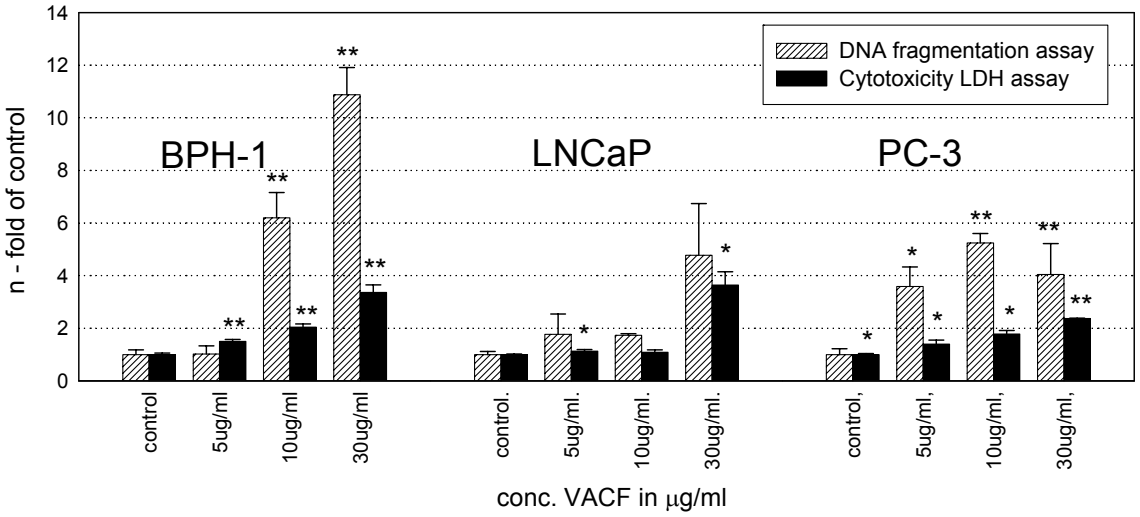


Figure III- 23 Effects of increasing doses of VACF on prostate cells DNA fragmentation and LDH release after 48h of treatment. Values were calculated as n-fold of control (n=1) and expressed as means ± SD (n = 3) (*,p<0.05 and **, p<0.01, compared to control).

Effects of VACL on DNA fragmentation and LDH release in human prostate cell lines

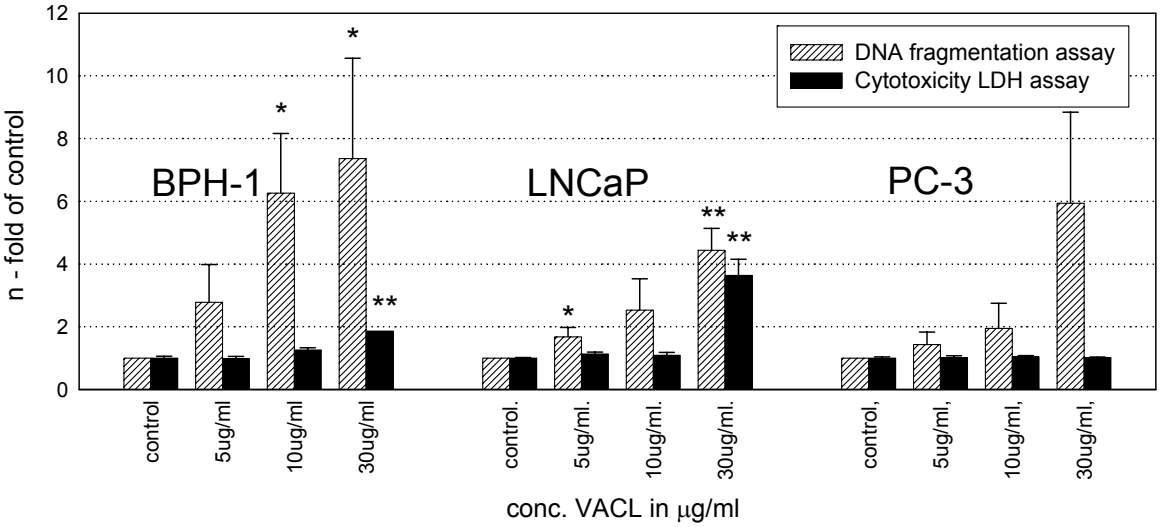


Figure III- 24 Effects of increasing doses of VACL on prostate cells DNA fragmentation and LDH release after 48h of treatment. Values were calculated as n-fold of control (n=1) and expressed as means ± SD (n = 4) (*,p<0.05 and **, p<0.01, compared to control).

3.3 Summary of the in vitro effects of SRE and VAC

Extracts of *Serenoa repens* fruits and *Vitex agnus-castus* fruits and leaves were tested for their effects on proliferating human prostate cancer cells. Investigated cell lines revealed to be less sensitive to SRE than to VAC treatment (Figure III-25). IC50 values obtained with all extracts investigated on all three prostate epithelial cell lines are summarized in Table III-4.

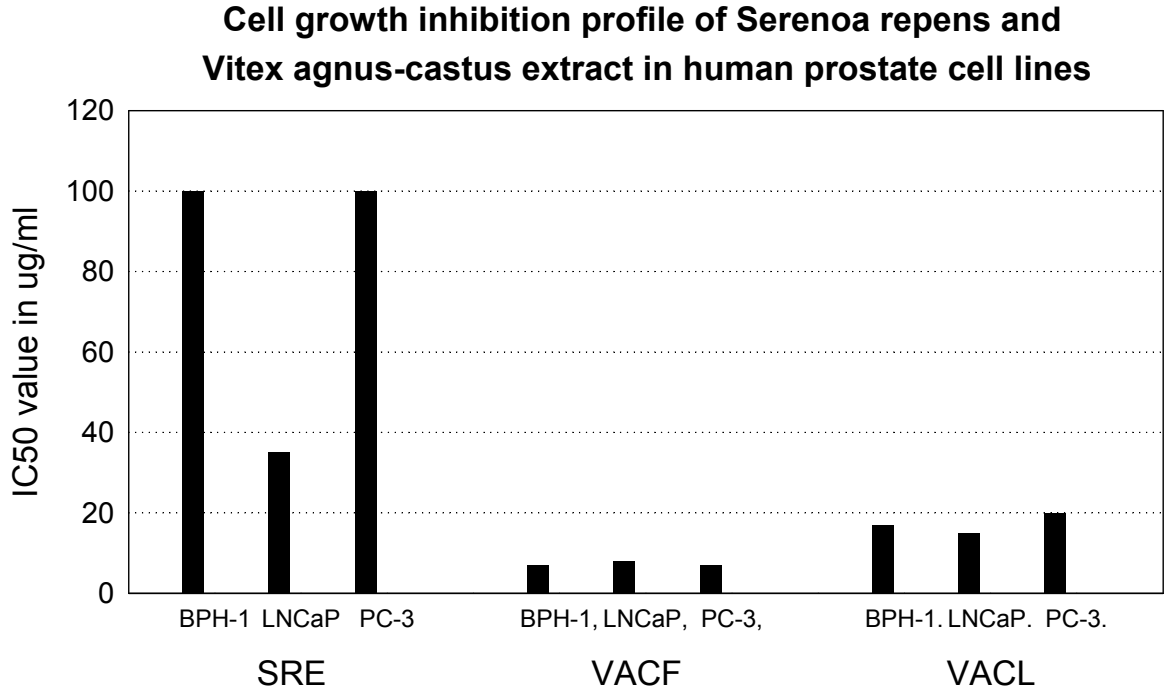


Figure III- 25 Growth inhibition profile of *Serenoa repens* and *Vitex agnus-castus* fruit and leaf extract in human prostate cell lines. After treatment with indicated extracts cells were fixed and stained with crystal violet. The optical density (O.D.) of the dissolved dye was determined at 550nm. IC50 values were expressed as % of controls (=100%).

Comparing the *Serenoa repens* effects in BPH-1, LNCaP and PC-3 cells, LNCaP cells revealed to the most sensitive cell line, showing growth inhibition with a 3-fold lower IC50 value than BPH-1 and PC-3. In LNCaP cells, SRE induced a mixed cell death phenotype, exhibiting apoptotic effects but also a low cytotoxicity, whereas in BPH-1 cells, SRE exhibited apoptotic effects without any cytotoxic effect, and PC-3 cells revealed to be almost insensitive to SRE treatment (Table III-4).

In contrast to SRE, VAC-induced effects appeared to be less specific for each cell line. Cell growth of all three cell lines was inhibited with similar IC50 values and exhibiting apoptotic effect but also low cytotoxic activity. The investigated fruit extract of VAC revealed to be the more potent cell growth inhibitor than the leaf extract with approximately two fold lower IC50 values (Figure III-25)

Table III- 4 Summary of the in vitro effects of SRE and VAC in human prostate cell lines

	Extracts		
	Me180	**VACF**	**VACL**
	IC50 values of prostate cell growth either with SRE and VAC for 48h		
BPH-1	~ 100 µg/ml	7 µg/ml	15 µg/ml
LNCaP	35µg/ml	8 µg/ml	17 µg/ml
PC-3	~ 100 µg/ml	7 µg/ml	20 µg/ml
	apoptotic pre-peak		
BPH-1	-	+	n.d.
LNCaP	+	+	n.d.
PC-3	-	+	n.d.
	partial reversion by pan caspase inhibitor		
BPH-1	+	+	+
LNCaP	+	+	-
PC-3	-	+	+
	DNA fragmentation		
BPH-1	+	+	+
LNCaP	+	+	+
PC-3	-	+	+
	cytotoxicity (LDH)		
BPH-1	-	+	+
LNCaP	+	+	+
PC-3	-	+	-

n.d. not determined

4 Discussion

4.1 The in vitro screening model system

4.1.1 Selected target cells

Benign prostatic hyperplasia (BPH) and prostate cancer (PCA) are known to be independent and intrinsically heterogeneous diseases. Of the hundreds of species of mammals, all of which have prostate glands, only humans and dogs have a propensity for spontaneously developing BPH and PCA (De Marzo et al 1999). Data obtained in rats and mice may not reflect the appropriate situations in men, because only the dorsolateral lobe of rodent prostates is ontogenetically comparable to the human prostate (Untergasser et al 1999). Thus, to assess the efficacy of potential chemopreventive or therapeutic agents by in vitro approaches, a promising strategy is to take advantage of cultured cells that broadly represent the spectrum of stages these proliferative prostate diseases are likely to exhibit. In this respect, in the present study, the panel of three different human prostate epithelial cell lines (BPH-1, LNCaP, and PC-3) was used for testing the effects of selected plant extracts.

BPH-1 cells were established from a BPH-specimen and may best resemble the BPH situation in situ. However, for the use in cell culture, the cell line was immortalised by SV40-large T antigen. It has been reported that SV40-large T antigen can bind to and inactivate p53 (Levine 1997). The tumour suppressor p53 is considered to play an important role in the control of the cell cycle, allowing DNA repairing or induction of apoptosis. Normal cells respond to DNA damage by increasing their production of p53. Mutations in the p53 gene, producing a defective protein, are often found in cancer cells (Schumacher et al 2001), but were also reported in BPH-1 tissue (Schlechte et al 1998).

PCA typically belongs to a class of tumours that are initially androgen hormone-dependent, but are well known to progress to hormonal therapy-resistant terminal stages (Klocker et al 1999). Therefore, LNCaP and PC-3 cells, the two other cell lines included in our cell model system, exhibit different growth properties that are best reflecting the intrinsic heterogeneity of human PCA. The LNCaP cell line is one of the most widely used in vitro model of prostate cancer. LNCaP cells maintain several characteristics of the primary human prostatic carcinoma, such as the

responsiveness to androgen (Andrews et al 2002b). They produce PSA, expresse the wild type p53 and a point mutation in the androgen receptor gene, which is also found in prostate cancer (Gaddipati et al 1994). They do not undergo apoptosis after androgen withdrawal, but respond to androgenic stimulation by proliferation (Lee et al 1995). By contrast, PC-3 cells are not responsive to androgens and do not express consistent levels of androgen receptor protein and are p53-negative (Mitchel et al 2000).

Considering all these characteristic features, our system appears to be a suitable in vitro model system for the screening of different plant extract with respect to their growth inhibitory activities in human prostate cells.

4.1.2 Assay systems

The applied assay systems are well accepted tools in preclinical drug development and therefore suitable methods for the evaluation of effects of plant extracts as an approach to rational phytotherapy.

Throughout the study, WST-8, a novel tetrazolium based assay, was used for cell viability assessment. This test has the advantage over previously used tetrazolium assays by producing a highly water-soluble formazan dye directly in situ (Ishiyama et al 1997). It is known from other tetrazolium salts (e.g. MTT) that their reactions are sensitive to the concentration of glucose, hydrogen ions, NADH, and NADPH (Vistica et al 1991). Since it is not described if the WST-8 is affected by any of these molecules, cell proliferation was verified by a second test using crystal violet which stains adherent cells. In the crystal violet staining assay the loss of cells due to manipulations is minimal, because of cell fixation in situ with 1% glutaraldehyd before any washing (Gillies et al 1986). As shown in Figures III-7 and III-17 (comparison of WST-8 –CV tested cells) the amount of formazan produced by viable cells is directly proportional to the number of living cells stained by crystal violet, indicating that the treatment did not reduce cell viability, and thus confirms the results obtained by the two assay systems.

Cell cycle analysis is a suitable method for the overall understanding of the influence of investigated drugs on cell cycle processes. However, our investigations revealed several problems associated with the cell cycle analysis. Defects in cell cycle control are some of the most common features of cancer cells, because they divide under

conditions under which their normal counterparts do not. In addition, polyploidy of cancer cells may exhibit discrepancies in cell cycle analysis. Furthermore, the mixture of constituents in our extracts may lead to several overlapping mechanisms preventing a clear dose-dependent single target effect. However, the subG1 “pre-peak” is a clear indicator for apoptotic fragmentation, which was further confirmed by other apoptosis-detection methods.

Apoptosis, “programmed cell death”, is a complex sequence of mechanisms which are functionally active at various time points, meaning that e.g. after short incubation times, DNA fragmentation may not be completed. Apoptosis is therefore preferentially not only assessed by a single method or at one time point. Hence, we investigated the co-treatment with a pan-caspase inhibitor interfering with several targets in the execution of apoptosis and further evaluated the extract-induced DNA fragmentation, a hallmark of late apoptosis. Since the Cell Death Detection ELISA used here does not require pre-labelling of cells, it can detect internucleosomal degradation of genomic DNA during apoptosis even in cells that do not proliferate. Unfavourable for our study, the test showed high standard deviations, which could possibly result from washing steps and small amounts of fragmented probes. However, the measured DNA fragmentation was in a comparable range in every fragmentation assay and was therefore a suitable method to complement the results from the cell cycle analyses and the co-treatment assays with the caspase inhibitor.

To complete our assay system, we additionally examined if the plant extracts displayed cytotoxic effects. The LDH Cytotoxicity Detection Kit is based on the measurement of the cytosolic enzyme lactate dehydrogenase (LDH) in the cell supernatants after release from damaged cells. The assay system was designed to allow simultanous measurements of LDH in the supernatants and DNA fragmentation in cellular extracts making a direct comparison of apoptotic and cytotoxic effects possible.

Our screening system revealed to be an appropriate screening system for the detection of the inhibitory activity of plant extracts in human prostate cell lines which were selected as cellular models of human proliferative prostate diseases.

4.2 In vitro investigations with extracts of *Serenoa repens*

4.2.1 *Serenoa repens*-induced cell growth inhibition

Primarily, the cell growth inhibiting effects of the commercially available *Serenoa repens* extract Me180 was assessed in three different human prostate epithelial cell lines representing different stages of prostate epithelium diseases. Proliferation of all three cell lines investigated was inhibited in a dose- and time-dependent manner by Me180-treatment (Figure III-5 and III-6). In particular, the androgen-responsive LNCaP cells were affected more severely by Me180 than the androgen-unresponsive PC-3 and BPH-1 cell lines, with respect to both cell growth inhibition and cellular damage (Figure III-6 and III-14). Me180 inhibited BPH-1 and PC-3 cell growth only at the highest concentrations (70-100µg/ml), while at lower concentrations little effect on cell survival was detected.

These observations are generally in line with results from others, who showed that *Serenoa repens* extracts (SRE's) were effective in reducing the survival of both androgen-dependent and independent human prostate cancer cell lines (Ravenna et al 1996; Iguchi et al 2001). Accordingly, both authors reported stronger effects in LNCaP cells than in other cell lines (PC-3 and others, unfortunately not specified). In the present study the cell growth inhibiting effects on LNCaP were 3-fold higher with IC50 values about 35µg/ml compared to 100µg/ml reported by Iguchi et al 2001. The discrepancies between these results may be partly due to the prolonged time of exposure from 24 to 48h, but could also be due to differences in the cell model system used. Investigating different exposure times we found that the Me180 induced a time-dependent growth inhibition over 72h (Figure III-5). However, in contrast to previous studies, the effects on LNCaP cells, even after 24h exposure time, were also more pronounced. A second explanation for these differences could be that we replaced the culture medium containing foetal bovine serum (FBS) with medium containing 1% charcoal-stripped serum (cFBS) devoid of steroid hormones and other factors, which could have a probable effect on cell growth. Ravenna and co-workers, who showed corresponding results in both LNCaP and PC-3 cells, reported medium change from 10% FBS to 5% cFBS (Ravenna et al 1996). In other reports investigating SRE on prostatic cell growth (Goldmann et al 2001; Iguchi et al 2001) no medium change was mentioned. Moreover, by evaluating the ideal serum

supplementation, we found that addition of 10% FBS medium did partly abrogate the growth inhibition effect, indicating that SRE could not overcome the mitogenic effect provided by growth factors contained in 10% FBS supplemented cell culture media (data not shown).

The fact that the androgen-responsive LNCaP cells were the most affected by the SRE treatment, may indicate that in some way hormone responsiveness makes cells more susceptible to the extracts. This provides further evidence, that the expression of androgen receptors and/or the responsiveness to androgens may play a crucial role in SRE-induced growth inhibition. Previous in vitro and in vivo studies have shown that the anti-androgenic effects may be related to an interference of SRE in several steps involved in the mechanism of action of androgens within target cells. In fact, some in vitro experiments on the rat ventral prostate tissue cultures and in cultured genital skin fibroblasts evidenced a significant inhibition of the binding of 5α-dihydrotestoterone (DHT) at nuclear and cytosolic receptor levels in the presence of SRE (Briley et al 1983; Briley et al 1984; Carilla et al 1984). In the same in vitro models, the drug caused a marked reduction in testosterone conversion to DHT and a partial inhibition of 3α-oxidoreductase activity followed by a decreased formation of 5α-androstane-3α-17β-diol (Briley et al 1984; Sultan et al 1984). Studies performed on baculovirus –directed insect cell expression system showed that SRE displayed inhibitory activity of both 5α-reductase isozymes type 1 and 2 (Delos et al 1995; Iehlé et al 1995). Estrogen and androgen receptor determination have been performed in the cytosol and in nuclear extracts from prostatic tissues of patients with BPH, confirming the anti-androgenic properties of SRE and evidenced its antagonist activity with nuclear estrogen receptors (Di Silverio et al 1992). Ravenna and co-workers further showed an SRE induced double agonistic/ weak antagonistic androgenic action that seemed to be mediated by the mutated androgen receptor in LNCaP cells (Ravenna et al 1996). It was reported further by the same authors that in the hormone-independent PC-3 cells, if transfected with the wild-type androgen receptor, SRE exerted a clear anti-androgenic responsiveness effect.

Taken together, all these findings may underline that hormone responsiveness makes cells more susceptible to the action of SRE. However, it can not be excluded that other factors may be involved. Our further investigations indicated that apoptotic, cytotoxic and anti-inflammatory effects must also be involved in determining the cell growth inhibition. We will consider these aspects in the next section.

4.2.2 Mechanisms of cell death induced by *Serenoa repens* extract

To further investigate the antiproliferative effects in more detail and to determine the mechanisms involved, cell cycle distribution, apoptotic and cytotoxic effects were examined after treatment with *Serenoa repens* extract Me180. Corresponding to the results obtained in cell growth assays, the treatment displayed the strongest dose-dependent effects in LNCaP cells in all investigations. In flow-cytometric cell cycle analysis Me180-treated LNCaP cells showed a dose-dependent increase of G0/G1 “pre-peak”, indicating an induction of apoptosis by the extract (Figure III-11). This observed apoptotic effect was evidenced by a dose-dependent increase of DNA fragmentation and a partial reversion of cell growth inhibitory effects of Me180 by co treatment with the pan-caspase inhibitor Z-VAD fmk (Figure III-13 and III-14). The detection of lactate dehydrogenase (LDH) in the tissue culture supernatant is an indicator of the toxicity of the compounds tested. The increase of LDH in the LNCaP cell supernatants with rising concentrations of Me180 shows, that at higher concentrations additional cytotoxic effects must also be involved in Me180 induced cell death, particularly at the highest concentration of 100µg/ml (Figure III-14).

In BPH-1 cells treated with Me180, the observed dose-dependent increase in DNA fragmentation (Figure III-14) and the dose-dependent partial reversion by the co treatment with the pan-caspase inhibitor (Figure III-13) was not accompanied by an increase of the apoptotic “pre-peak” in cell cycle analysis (Figures III-10). These results appear contradictory but might be explained as follows: The lack of an apoptotic “pre-peak” indicates the lack of small DNA fragments. However, the presence of a sub-diploid peak (sub G2/M, larger DNA fragments) is a second indicator of apoptosis (Darzynkiewicz et al 2001). Cell cycle analysis of BPH-1 cells after treatment with Me180 revealed a shift from G1 to S phase (Figure III-10). It was not possible to distinguish a clear “sub-diploid” G2/M peak, but we suggest that the enhanced DNA fragmentation, measured by DNA fragmentation assay, is due to larger DNA fragments found in the enhanced S phase.

In PC-3 cells, in accordance with the minor cell growth inhibition, no apoptotic or cytotoxic effects could be assessed after the treatment with Me180. Cell cycle analysis revealed a slight dose-dependent but non significant decrease of the percentage of cells in G1 phase with a shift to S phase. However, this effects could not be explained by an apoptotic effect due to the lack of an increase of DNA

fragmentation and in the partial reversion with the pan-caspase inhibitor co-treatment, indicating that the PC-3 cells are almost insensitive to SRE treatment (Figures III-13 and III-14).

SRE exhibited a dose-dependent apoptotic effect in LNCaP cells, and, surprisingly, also in BPH-1 cells, whereas PC-3 cells were insensitive. Therefore, the extract possibly exerts these effects to cell lines at target sites present in both cell lines such as p53 which is lacking in PC-3 cells. p53 was reported to mediate G1 arrest, but has also been implicated in G2/M phase checkpoint (Levine 1997). However, cell cycle analysis of SRE treated BPH-1 and LNCaP cells did not show any significant dose-dependent changes in cell cycle distribution of these phases (Figures III-10 and III-11). Furthermore, it remains to be seen why the apoptotic effect did not result in a more pronounced cell growth inhibition in BPH-1 cells.

Our data at least partly confirm results from fluorescence microscopy studies by Iguchi et al (2001), that the treatment of SRE induced mixed (apoptotic and necrotic) cell death involving caspase activation. Additionally, in a first report by Bayne and co-workers, morphological changes were also observed in a co-culture model of BPH (epithelial and fibroblast cells (10:1) of human BPH tissue), including the polarisation of the nuclei and condensation of chromatin following treatment with SRE (Bayne et al 1999). These data suggested an induction of apoptosis in both the epithelial and stromal cell of the prostate. Furthermore, an increase of the apoptotic indices of prostate epithelial cells and stromal cells in vitro was detected by the same authors (Bayne et al 2000). These effects were further confirmed in prostate tissues of BPH-patients by Vacherot and co-workers showing that after three months of SRE-treatment the imbalance in the apoptosis/proliferation ratio was only reversed in treated but not in the untreated patients (Vacherot et al 2000). Another recent study reported the shrinkage of the epithelial tissue in the transition zone of the gland after six months of SRE-treatment (Marks et al 2000).

Taken together, all these data underline the possible reversion of the imbalance between proliferation and apoptosis in progressive prostatic diseases by SRE extracts. However, in clinical trials the size of enlarged prostates was only weakly reduced by SRE after treatment for six months (Carraro et al 1996) or twelve months (Debruyne et al 2002). Nevertheless, in both studies, a further time-dependent

enlargement was not observed, indicating that the extract could at least exhibit a preventive action of a further enlargement of the prostate.

In addition, SRE has so far rarely been investigated on prostate carcinoma specific models. Interestingly, Ishii and co-workers reported that the invasive activity of PC-3 cells into Matrigel was effectively suppressed by low doses of 10µg/ml of a SRE (Ishii et al 2001). They further showed that the purified urokinase-type plasminogen activator (uPA) activity was inhibited by SRE in a dose-dependent manner, suggesting that the suppression of PC-3 cell invasion by the extract is based on an inhibition of the uPA activity which is necessary for tumour cell invasion. SER was therefore proposed as a possible useful agent for the therapeutic treatment of prostate cancer. Moreover, it was suggested in a further study on prostate cancer cells (Goldmann et al 2001) that reduced cellular growth after SRE treatment may be related to decreased expression of COX-2 and may further be due to down-regulation in the expression of Bcl-2. Since increased COX-2 expression is associated with an increased development of prostate cancer, SRE was further proposed as a possible preventive prostate cancer treatment (Goldmann et al 2001).

4.2.3 Anti-inflammatory activity of *Serenoa repens* extracts

Frequently BPH is associated with a chronic inflammation that results from infiltration of inflammatory cells into the prostate and this inflammation is one of the aetiological factors involved in the development of BPH (Theyer et al 1992). Production of prostaglandins such as prostaglandin E_2 (PGE_2) and leukotrienes (leukotriene B_4, LTB_4) are potent inflammatory mediators and may contribute to the development of the disease. It was shown in a contemporaneous diploma thesis by Hubacher, that Me180 inhibited the activity of recombinant COX-1 and COX-2, as well as the activity of 5-LOX in differentiated HL-60 cells, with IC50 values of 6µg/ml, 30µg/ml and 12µg/ml, respectively (Hubacher 2002). These results suggest that by inhibition of these enzymes the production of these inflammatory mediators is reduced after Me180 treatment. Our findings that Me180 inhibits COX-1, COX-2 and 5-LOX activity are consistent with previously reported investigations. Breu and co-workers showed a dual inhibitory activity of recombinant COX and LOX enzyme activity (Breu et al 1992). Furthermore, Paubert-Braquet and co-workers showed that SRE significantly inhibited the production of several 5-lipoxygenase metabolites (5-HETE, 20-COOH

LTB_4, LTB_4 and 20-OH LTB_4) at concentrations as low as 5 µg/ml in human polymorphonuclear neutrophils (Paubert-Braquet et al 1997).

In this context several studies were conducted with patients treated with SRE. Helpap and co-workers found a significant reduction in periglandular stromal edema, intraglandular congestion and congestive prostatitis but no changes in the epithelium from patients treated for 12 weeks with SRE as compared to placebo (Helpap et al 1995). In a very recent pilot study by Vela Navarrete and co-workers a 3-month treatment with the SRE produced a significant reduction of the cytokines IL-1β and TNF-α, concomitant with a reduction of prostatic interstitial B lymphocytes and a significant clinical improvement (Vela Navarrete et al 2003). The authors speculated that SRE modifies the inflammatory status of BPH tissue through this cytokine regulation.

Beyond this positive effect in BPH, these anti-inflammatory effects are also important in the context of the cancer preventive effects by non-steroidal anti-inflammatory drugs (NSAIDs) (Steele et al 2003). It was shown that PGE_2 is produced in greater amounts in BPH than the normal tissue from which they were derived (Chaudry et al 1994). Moreover, several studies have also described increased COX-2 tissue expression in PCA (Steele et al 2003). As a group, non-steroidal anti-inflammatory drugs (NSAIDs) and particularly COX-2 inhibitors have been shown to have antiproliferative effects in BPH-1, LNCaP and PC-3 cells, by increasing the rate of apoptosis (Lim et al 1999; Hsu et al 2000; Andrews et al 2002a). Additionally, it was previously reported that inhibition of 5-lipoxygenase triggers massive apoptosis in both androgen-sensitive (LNCaP) and androgen-refractory (PC-3) human prostate cancer cells (Ghosh & Myers 1998). This leads to the assumption that the SRE by inhibiting COX-2 and 5-LOX activity can contribute to the apoptotic effects observed in LNCaP and BPH-1 cells in our study. However, further studies will be needed to clarify the involvement of these enzymes in cell growth inhibition by the extract.

Taken together, these investigations show, that SRE may provide beneficial effects in concomitant inflammatory conditions in BPH but also act as a possible preventive agent against PCA.

4.2.4 Comparison of various *Serenoa repens* extracts with respect to cell growth inhibition in prostate cell lines

It was further evaluated, if the ripeness condition and solvents used for extraction has an influence on the quality of the extracts with respect to their antiproliferative properties in the three prostate cell lines. No relevant differences were found among the extracts studied with respect to cell growth inhibitory effects (Figure III-8 and III-9). IC50 of hexane extracts were found to be slightly lower (31µg/ml) than the ethanol extracts (35µ/ml), however these differences were statistically not significant ($p>0.05$). This lack of differences was in fact somewhat surprising. Extracts are mixtures of various constituents. Ripeness conditions and the solvent used for extraction may affect the final constituent composition. These parameters should therefore not be neglected when investigating plant extracts in in vitro models. Our results suggest that the active – cell growth inhibiting constituents must be present in all extracts in similar amounts, independent of ripeness condition whether with hexane or ethanol as solvents. However, so far, our extracts investigated were not studied analytically.

Differences in cell growth inhibitory activities by different extracts could lead to further extract optimisations. Further investigations such as selective fractionation and tests with single compounds are needed to evaluate the responsible constituents and constituent groups involved in the observed actions, to allow a selection of the best (most effective and less toxic) extract for treatment. It was reported in previous studies that the anti-androgen (5α-reductase inhibitory activity (Weisser et al 1996; Raynaud et al 2002)) and anti-inflammatory (Breu et al 1992) effects were found in the lipophilic saponifiable subfraction consisting mainly of oleic acid (33%), lauric acid (24%), myristic acid (11%), and palmitic acid (9%) (Breu et al 1992). Moreover, myristoleic acid was identified as one of the active components of the extract, exhibiting a mixed apoptotic/necrotic action (Iguchi et al 2001).

In our in vitro cell model system no relevant differences could be found regarding cell growth inhibitory activity. However, it is unknown, if ripeness condition or extract solvent may exert any influence on the activity of the extracts in other model systems (e.g. stroma cells) or in vivo.

4.2.5 In vitro results as indicators for activities in vivo

In vitro studies, which represent antiproliferative effects following a constant drug exposure against rapidly growing cell lines as well as tests on recombinant enzymes, cannot be directly projected to effects in vivo. At present, the pharmacodynamic effects of our in vitro findings cannot be directly extrapolated to the clinical application since the concentration of biologically available SRE in the human prostate is unknown. However, they can indicate the possible mechanisms of action involved. Furthermore, as mentioned before, our data were confirmed by previously reported in vitro results from others using different SREs. The predicted physiological concentration of SRE, assuming distribution in total body fluid achievable using the recommended therapeutic dosage was reported to be 10-30µg/ml (Bayne et al 1999). It should be pointed out that the highest concentration of *S.repens* extract in our investigations was 100µg/ml. Due to solubility problems, the examination of higher concentrations was impossible. It was reported that using oral administration of ^{14}C-labeled oleic and lauric acids (two major components of SRE) in rats, the radioactivity selectively accumulated in the rat prostate gland (Chevalier et al 1997). In addition, when the effects of a SRE was investigated on human primary cultures of fibroblasts and epithelial cells from the prostate, epididymis, testes, kidney, skin and breast, a selectivity was found for prostate cells, indicating that the plant extract can be expected to be organ selective. In this study, the morphological changes in the prostate were accompanied by an increase in the apoptotic index in the prostate cells along with an inhibition of the activity of the nuclear membrane bound 5α- reductase isoenzymes. No such changes were observed in any of the other cells studied (Bayne et al 2000).

4.2.6 Conclusion on the effects by *Serenoa repens* extracts

In conclusion, these results indicate that extracts of SRE may retard human prostate cell growth by multiple mechanisms, including hormone-dependent interaction, apoptosis, and inhibition of COX1, COX-2 and 5-LOX but also by a low cytotoxic action. These possible mechanisms may have beneficial effects on the one hand in symptomatic BPH and associated inflammation, and on the other hand, the observed apoptotic effect and the inhibition of COX-2 and 5-LOX may also contribute to a possible effect in prostate cancer prevention.

In the present studies we found that the commercially available extract Me180 exerted similar effects in vitro as other SRE previously investigated and that variation in ripeness conditions and the choice of solvents (hexane or ethanol) exert no crucial differences in potency on growth inhibitory activities.

The exact mechanism of SRE in prostatic epithelial cell proliferation, however, remains elusive and needs to be further investigated, by e.g. fractionating the extracts to isolate active constituents. Since SRE is a more potent inhibitor of the growth of androgen-responsive prostate cells such as LNCaP, further studies should be performed to establish whether the action observed is related to androgen and estrogen receptor expression or to the apoptotic and inflammatory enzyme inhibiting effects observed.

4.3 In vitro investigations with extracts of *Vitex agnus-castus*

4.3.1 *Vitex agnus*-castus –induced cell growth inhibition in prostate cell lines

The present study provides a new insight into the mechanism of action of *Vitex agnus-castus* extracts. VACF are described to have a beneficial effect on disorders related to hyperprolactinaemia such as cycle disorders, luteal phase defect, mastodynia and premenstrual syndrome (Wuttke et al 2003). Recently, a VACF was shown to exhibit anti-tumour activities in different human cancer cell lines (Ohyama et al 2003), but its effects on human prostate epithelial cells has so far not been investigated.

The proliferation of all three prostate cell lines was effectively inhibited in a dose- and time-dependent manner with IC50 values below 10µg/ml for *Vitex agnus-castus* fruit extract (VACF) and ~ 20µg/ml for *Vitex agnus-castus* leaf extract (VACL), respectively. All three cell lines were affected by the treatment in a comparable manner, without showing any cell specificity (Figure III-16).

These findings prompted us to further investigate the mechanisms of action of these extracts at the cellular level. In contrast to cell proliferation, cell cycle analysis revealed a cell line specific alteration in the progression of cell growth. In all three cell lines, VACF treatment showed a dose-dependent, statistically significant increase of the apoptotic “pre-peak” (Figures III-18-20). Moreover, VACF-treated BPH-1 cells exhibited a significant dose-dependent G2/M arrest associated with a decrease of cells in G0/G1 phase (Figure III-18). It remains to be determined whether the ability of VACF to arrest cells in the G2/M phase is modulated by cyclin dependent activities, such as by cdc2-cyclin B kinase. The G2/M DNA damage checkpoint prevents cells from entering mitosis if the genome is damaged and the cdc2-cyclin B kinase is pivotal in regulating this transition (Ohi & Gould 1999). Beyond the apoptotic “pre-peak”, cell cycle analysis of VACF treated LNCaP cells did not display any further significant cell cycle arrest (Figure III-19). In PC-3 cells the dose-dependent and statistically significant ($p<0.01$) increase of cells in S phase may be a second evidence of an increase of apoptotic bodies, since the presence of a sub diploid peak (sub G2/M) is a second hallmark of apoptosis (Darzynkiewicz et al 2001).

Cell cycle analysis did partially reveal a significant dose-dependent shift in cell cycle distribution. However, the mixture of compounds in extracts may have several different mechanisms, leading to complex changes in cell cycle distribution. Extract fractionations and the investigation of single compounds may lead to a better understanding of the mechanisms involved. Of particular relevance are the molecular mechanisms of cell cycle regulation involving cyclins, and cyclin dependent kinases linked to specific cell cycle phases, such as CDK4/6-cyclin D and CDK2-cyclin E controlling the G1/S phase checkpoint (Boonstra 2003) and cdc2-cyclin B kinase controlling G2/M phase checkpoint (Ohi & Gould 1999).

The dose-dependent increase of sub G0/G1 (apoptotic) peak in all three cell lines suggested that part of the cells were undergoing apoptosis. This observed induction of apoptosis was confirmed by a dose-dependent enhancement of DNA fragmentation in the cytoplasm of cells after treatment with the extract (Figure III-23, III-24). Moreover, this interpretation was supported by the partial reversion of the growth-inhibitory effects in presence of a pan-caspase-inhibitor (Z-VAD-fmk) clearly indicating that caspases must be involved in the underlying mechanism (Figures III-21, III-22). Taken together, these results suggest that the cell death induced by VACF and VACL proceeds, at least partly, via an apoptotic mechanism. However, the increased lactate dehydrogenase (LDH) activity in supernatants of VACF-treated cells, measured as a marker of cell necrosis, indicates additional cytotoxic effects with increasing doses of VACF and VACL (Figures III-23, III-24).

It was previously reported that a *Vitex agnus-castus* fruit extract exhibits growth inhibitory activity in several cell lines (Ohyama et al 2003). The authors suggested that the cytotoxic activity may be attributed to the growth activity of the respective cells and that the cytotoxicity depended on the phase of cells in the cell cycle. This interpretation can not be confirmed by our investigations. Firstly, although our three investigated cell lines exhibited different cell growth rates, the VACF-induced cell growth inhibition was comparable in all three cell lines with IC50 values of 7, 8, and 7 ug/ml, respectively (Figure III-16). Secondly, in our test system, cells of all three cell lines passed at least one cell cycle during the investigated treatment time of 48h. Therefore, the cell growth inhibitory activity cannot only depend on the phase of cells in the cell cycle. Moreover, if the chosen treatment time is too short for an entire cell cycle passage, such an effect can only be recorded and assessed in synchronized cells. Without prior synchronisation, results from a mixed cell population can lead to

misinterpretation. However, in agreement with the investigations by Ohyama and co workers we found that VACF induces cell growth inhibition partially by an apoptotic pathway and that caspases are involved.

Our studies further revealed that Vitex agnus-castus LEAF extract exhibited weaker growth inhibitory effects than the FRUIT extract. It was shown by Huber (2002) that except for a higher quantity of hydrophobic flavonoids in leaves, the thin layer chromatographic fingerprints of *Vitex agnus-castus* fruit and leaf extracts of the same plant were very similar. Therefore, leaf extracts were investigated in order to test its suitability as an alternative raw material, since leaf extracts would have the advantage that the material could be harvested from plants growing in Switzerland, in contrast to fruits that are only found in higher amounts in Mediterranean regions.

Moreover, further investigations with single compounds should be performed to examine which agents are responsible for the growth inhibitory activity. Two single compounds, rotundifuran, a labdane diterpene, and luteolin, a flavonoid present in *Vitex agnus-castus* extracts were reported to induce apoptotic effects in HL-60 cells (Ko et al 2001, 2002). A third agent, agnuside (an iridoid glycoside), showed preferential inhibition of COX-2, while having only small inhibitory effects on COX-1 (Suksamrarn et al 2002). The cell growth inhibitory activities of VACF against prostate cell lines may be attributed to these compounds. Further investigations with extract fractionations and single compounds are needed to find the active components.

4.3.2 Potential beneficial effects of *Vitex agnus-castus* in prostate diseases

VACF are described to have a beneficial effect on disorders related to hyperprolactinaemia such as cycle disorders, luteal phase defect, mastodynia and premenstrual syndrome (Wuttke et al 2003). The rationale for the investigations of VACF in our prostate screening model system was fourfold:

- VACF induced cell growth inhibition in several human cell lines
- Inhibition of binding to dopamine D2 and D3 receptors and inhibition of prolactin secretion
- COX-1, COX-2 and 5-LOX inhibitory action in vitro
- Estrogen receptor β selectivity action in vitro

Our investigations revealed several potential uses of VAC in prostate diseases.

Both BPH and PCA are typical slowly progressing diseases. Rates of PCA cellular proliferation of 3.0% and below are typical (Cher et al 1995), making this one of the slowest growing malignancies known. Indeed, this low growth rate of PCA may be responsible, at least in part, for the weak efficacy of cytotoxic agents, which interfere with DNA synthesis. Chemotherapeutic drugs currently available in clinical use preferentially inhibit the proliferation of cancer cells (Tang & Porter 1997). Thus, it can be expected, that patients with slow-growing prostate cancer cells will not respond to most of antiproliferative chemotherapeutics. Therefore, extracts that do not affect cancer cell proliferation or cell cycle progression because of their apoptosis-inducing effects may have beneficial effects in this indication.

By demonstrating that *Vitex agnus-castus* induces cell growth arrest and apoptosis in PCA cells apparently through different mechanisms of action, *Vitex agnus-castus* appears to be a potential anti-prostate-cancer agent. Additionally, the complex mixture of phytotherapeutics, composed of different compounds may exhibit several pharmacological properties, accounting for their potential multiple mechanisms of action. Moreover, these multiple actions may possibly prevent the development of drug resistant tumours.

Our in vitro experiments displayed new, interesting activities; it is however too early to extrapolate directly to clinical applications. Further investigations are needed to

gain further insights into the molecular mechanisms of cell death involved and to evaluate the potential effects on normal prostate cells. VACF was shown to be safe in various clinical studies, leading to the conclusion that the cytotoxicity cannot be very prominent. To our knowledge, the extracts were so far never clinically investigated in men. The interesting activity profile of VACF in our in vitro system points to potential new therapeutic targets also in men.

Role of prolactin in the control of prostate growth:

The mechanism of action in diseases related to hyperprolactinemia was mainly explained by the prolactin (PRL) lowering effect via a dopaminergic action (Schellenberg 2001; Wuttke et al 2003). PRL is widely expressed in different tissues, and it is one of several polypeptide factors known to exert trophic effects on the prostate. In males it is known to influence reproductive functions and it is well-established that PRL stimulates the proliferation and differentiation of prostate cells (Reiter et al 1999). PRL, alone or synergistically with androgens, plays physiologically significant roles in the normal prostate (Reiter 1999). Moreover, it was shown that PRL acts as a direct growth and differentiation factor for human prostate, as indicated by changes in DNA synthesis and epithelial morphology of organ cultures (Nevalainen et al 1997). In addition, it was demonstrated by the same authors that human and rat prostate cells synthesize PRL. Thus, this hormone may regulate prostate growth by an autocrine/ paracrine loop.

However, the potential role of PRL in proliferating prostate dieases, BPH and PCA, are very conflicting and need to be further defined. Controversies exist concerning an increase of circulating PRL levels with age (Vekemans & Robyn 1975; Hammond et al 1977). Additionally, conflicting results have been reported, showing either an increase in PRL levels (Saroff et al 1980; Odoma et al 1985), or unchanged levels of this hormone in the case of BPH and PCA (Harper et al 1976; Hammond et al 1977; Lissoni et al 2000). These differences could be partially explained by the recent observation that PRL is produced in the prostate itself (Reiter et al 1999).

Moreover, the expression of PRL receptors in the prostate cell lines used in our study was controversially discussed: It was reported by Untergasser and co-workers that in contrast to BPH and PCA samples, the used prostate cell lines used in our model system (BPH-1, LNCaP, PC-3) do not express PRL receptors (Untergasser et al 1999). In contrast, it was recently reported that PRL receptors were expressed in

LNCaP and PC-3 cells but despite this, PRL had no significant effect on proliferation (Ruffion et al 2003).

Taken together, the potential role of PRL in prostate is not fully understood, but a proliferative activity seems to be assured. The PRL pathway has not yet been taken into consideration for prostate diseases and its role in development of BPH and PCA is still an issue that needs to be further addressed. Thus, a potential interference of PRL on proliferative effects of the prostate in BPH and PCA by *Vitex agnus-castus* extract has to be further investigated.

COX-1, COX-2 and 5-LOX inhibiting action in vitro

At the present time, the precise mechanisms by which VAC exerts its growth inhibitory, apoptotic, and cytotoxic effects are not known. It was shown in a recent diploma thesis by Hubacher that VACF inhibited COX-1 and COX-2 and 5-LOX by blocking the synthesis of eicosanoids such as prostaglandin E_2 and leukotriene B_4 with IC50 values of 30, 40, and 35 µg/ml, respectively (Hubacher 2002). The eicosanoids are known to play a critical role in cancer cell biology. They facilitate cancer transformation by stimulation of cell growth, tumour progression and immune suppression (Pruthi et al 2003). Formation of eicosanoids have been implicated in the action of a number of cytokines, and growth factors e.g. epidermal growth factor, platelet derived growth factor, and bombesin. The specific eicosanoids responsible for mitogenesis of prostate cancer cells included prostaglandin E_2 (PGE_2) as well as several lipooxygenase products. In human prostate tissues, PGE_2, is the only significant eicosanoid produced (Chaudry et al 1994). Previous studies by Tjandrawinata and co-workers demonstrated that dimethylPGE2 stimulates cell growth of PC-3 and LNCaP cells (Tjandrawinata et al 1997). In PC-3 cells COX-2 mRNA level was increased by PGE_2, while the NSAID flurbiprofen (5µM) inhibited the up-regulation of COX-2 mRNA expression as well as PGE_2 mediated stimulation of PC-3 cell growth. Moreover, it was shown in a in vivo study (Gupta et al 2000) that COX-2 is over-expressed in prostate cancer. The authors demonstrated that COX-2 mRNA in prostate cancer was 3.4-fold higher in the non-cancerous tissue and that COX-2 protein was over-expressed in 83% of the tumour samples. In addition, the selective inhibition of 5-lipoxygenase (5-LOX) by the lipooxygenase inhibitor MK886 was shown to completely block 5-HETE production and to induce massive apoptosis in both LNCaP and PC-3 cells (Ghosh & Myers 1998).

Another possible mode of action is the ability of both COX enzymes to deplete the substrate arachidonic acid. COX over expression depletes cellular arachidonic acid levels and correspondingly free arachidonic acid substrate. The increased level of arachidonic acid could result in activation of sphingomyelinase, which can cause increased levels of ceramide, a compound that also can induce apoptosis (Chan et al 1998). However, specific COX-1, COX-2 and 5-LOX protein level measurement or the measurement of ceramides are needed to determine if the cyclooxygenase or LOX pathway is responsible for the growth inhibitory and apoptosis inducing activity observed with the extract.

Several COX-2 inhibitors were shown to have anti-tumour activities in prostate cancer (Chan et al 1998; Cao & Prescott 2002; Pruthi et al 2003). COX-2 is highly expressed in all three cell lines (Lim et al 1999; Hsu et al 2000). Therefore, the COX-2 inhibition found with isolated enzymes could give an explanation for the observed apoptosis in prostatic cell lines in our test system. It remains to further investigate, if VAC exhibits any effect on COX-2 expression and/or PGE2 production at the cellular level.

Estrogen β receptor selectivity action

Previous studies reported of estrogenic effects of fruit and leaf extract with the leaf extract being more active than fruit extracts (Berger 1998). Investigations on estrogen receptor subtype -α and –β revealed VACF induced inhibition with selectivity to the subtype β (Jarry et al 2003). Estrogen receptor β (ER-β) is preferentially localized in the prostate epithelium (Chang & Prins 1999). ER-β knockout mice exhibit prostatic hyperplasia and this animal model favours an antiproliferative role of ER-β (Weihua et al 2002). In this regard it was proposed that ER-β is a potential target of therapeutical intervention in proliferative prostatic disease. ER-β were reported to be highly expressed in our three cell lines investigated (Lau 2000). Therefore, induction of cell growth inhibition through binding of VAC to ER-β would be a further possible mechanism and deserves to be further investigated.

4.3.3 Conclusion on the effects of Vitex agnus-castus in prostate cells

In summary, these studies provide the first evidence that fruit and leaf extract of *Vitex agnus-castus* are effective inhibitors of the growth of human prostate cancer cells by inducing apoptosis, but also by inducing a low cytotoxic effect. Comparing the effects of VAC fruit with leaf extract, the fruit extract revealed to be two fold more potent than the leaf extract. The VAC-induced cell growth inhibition was not cell type specific and a mixed apoptotic/cytotoxic cell death must be involved in all three cell lines investigated. However, cell cycle analysis revealed the mechanism involved may vary among the cells indicating that possibly more than one compound might be responsible for the observed effects. Moreover, before studies on its possible role in clinical use will be performed, VAC effects in normal prostate cells have to be examined.

To our knowledge, these preclinical investigations were the first to examine the possible impact of *Vitex agnus-castus* fruit and leaf extracts in proliferative prostatic diseases. Our results suggest that extracts of VAC by modulating cell cycle and apoptotic machinery as well by inhibiting inflammatory enzymes may possess a potential for development as an agent for prevention and/or therapy of benign and malign prostatic hyperplastic diseases. Moreover, further development could lead to an extract modification, producing special extracts with stronger apoptotic activities, but with weak or no cytotoxic activities. Detailed studies are also required to further clarify their mechanisms of action and to investigate their effects in vivo.

5 General conclusion and outlook

Our in vitro studies revealed to be a representative model for future screening of extracts for the rational use of phytotherapeutic drugs in proliferative prostate diseases. The data documented that the extracts of the two plants chosen, differed widely in their ability to inhibit prostate cell growth and in the mechanisms involved. Agents that are capable of specifically inducing apoptosis of tumour cells are desirable in developing novel therapeutic and preventive approaches. However, their effects on normal prostate cells remain to be investigated.

BPH and PCA are heterogeneous diseases. In the development of BPH, epithelial and stromal factors are involved. Thus, our observations should be confirmed, e.g. by a co-culture cell system investigating epithelial and stromal cell interactions, to better represent the situation of human BPH tissue. As the toxicity of both extracts has already been widely assessed in clinical trials (for VACF with other clinical applications), and estimated to be safe (Wilt et al 2002; Wuttke et al 2003), the accurate model systems are still tissue investigations of patients and healthy volunteers after treatment with the extracts.

The *Serenoa repens* extract Me180 induced a cell-specific stronger effect in androgen-responsive LNCaP cells than in androgen-unresponsive BPH-1 and PC-3 cells, indicating that a hormone-related mechanism may be involved. In LNCaP cells Me180 induced both apoptotic but also cytotoxic effects, whereas in BPH-1 cells, Me180 induced weak apoptosis without exhibiting cytotoxicity and PC-3 cells were insensitive to the treatment. It was previously shown that Me180 exerts an inhibitory activity on COX-1, COX-2 and 5-LOX enzymes (Hubacher 2002). Therefore, it remains to be investigated, if the inhibitory activities mainly observed in LNCaP cells are linked to an inhibitory activity towards these enzymes. This could be either tested by the measurement of the production of prostaglandins such as prostaglandin E_2 (PGE_2) and leukotrienes (leukotriene B_4, LTB_4) produced by these cells or by quantification of the expression of COX-2 and 5-LOX in treated cells versus control cells.

Moreover, since Me180 is a more potent inhibitor of the growth of androgen-responsive prostate cells LNCaP, further studies should be performed to establish whether the action observed has an impact on androgen and estrogen receptor

expression and its correlation to the apoptotic and anti-inflammatory effects observed.

It was further evaluated, if the ripeness condition and solvents used for extraction changed the activity of SRE on proliferation properties of the three cell lines. With respect to cell growth inhibitory effects, no relevant differences were found among the extracts studied. Further investigations such as selective fractionation and tests with single compounds will be needed to find the responsible constituents or constituent groups involved in the observed actions to allow a selection of the best (most effective and less toxic) extract for the treatment.

When comparing SRE and VAC, the latter was the more potent inducer of cell growth inhibition (Figure III-25). In addition, no cell specificity was shown in growth inhibitory effects of VAC. Comparing the effects of VAC fruit with leaf extract, the fruit extract revealed to be two fold more potent than the leaf extract. Exposure of BPH-1, LNCaP, and PC-3 to VAC induced characteristic features of apoptosis, including an increase of DNA fragmentation confirmed by a G1 “pre-peak” in cell cycle analysis, and the involvement of caspases action as cell growth inhibition was partly reversed by a pan-caspase inhibitor, but VAC also exhibited a low cytotoxic effect. However, first of all, its effect on normal prostate cells has to be investigated, since an effect on normal cell growth would not always be a desirable effect. Moreover, the described in vitro effects of VAC have to be addressed by means of additional cellular and molecular studies:

The interference with the apoptotic machinery needs to be further elucidated. We have evidence to expect, that caspase-induction must be involved. The pan-caspase inhibitor Z-VAD-fmk does not distinguish among the numerous caspases involved. Therefore, specific caspase inhibitors could trace the exact pathway of cell death involved. Cell cycle analysis did partially reveal significant dose-dependent interference with the cell cycle distribution. Extract fractionations and the investigation of single compounds could lead to clearer results and to a simplification of the interpretation. Of particular relevance are the molecular mechanisms of cell cycle regulation involving various mitogen activated kinases, cyclins, and cyclin dependent kinases linked to the specific cell cycle phases. Additionally, more detailed studies to explore the genetic and signal transduction pathways should be addressed. BPH epithelial cells overexpress bcl-2, an antiapoptosis protein that could be involved in

the decrease of apoptosis in human BPH (Kyprianou et al 1996; Colombel et al 1998). Furthermore, the involvement of COX and LOX enzymes and their association with cell growth inhibitory activities need to be investigated in more detail. This is an area worth additional investigations to further elucidate the mechanism of action of VAC in the human prostate gland.

Consistent with previous reports of SRE, and shown for the first time with VAC, the exhibited cell growth inhibition in human prostate epithelial cells is preferentially due to apoptosis, although a low cytotoxic effect was also detected in both extracts. Our investigations underline the possible reversion of the imbalance between proliferation and apoptosis in progressive prostatic diseases by both extracts, however, by different mechanisms.

The call for the development of more effective, more selective, and less toxic agents in age-related proliferative prostate diseases has become more urgent with the increasing age of the male population. One approach is the rational targeting of processes unique to BPH and PCA, respectively. Both diseases are uniquely recognised as multifactorial and slow-developing processes. Because of its high incidence and its long latency period, BPH and PCA are ideal targets for chemoprevention and therapy by phytotherapeuticals. Moreover, epidemiological studies show considerable geographical variation in the age-adjusted incidence of BPH and PCA (Moller-Jensen et al 1990). While some dietary constituents are implicated in cancer and BPH promotion, increasing evidence suggests that certain plant derived nutrients can suppress tumour development (Griffiths et al 1999). Therefore, phytotherapeuticals may supply further interesting means for preventive and therapeutic medication.

Further studies have to assess if the in vitro results have any clinical relevance in vivo. The safety of both extracts was investigated in a few clinical trials, although for VACF in another clinical application, and they considered to be safe (Wilt et al 2002; Wuttke et al 2003). A reliable assessment of efficacy can only be stated by a placebo or at least reference controlled clinical trial, since only randomised controlled trials can provide sufficient evidence for effectiveness. Thus, only a rational phytotherapy based on this knowledge, can be considered as adequate for prevention or treatment in proliferative prostate diseases.

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IV CLINICAL INVESTIGATIONS

A controlled, double blind, randomised, parallel-group pilot study

to compare safety, tolerability and therapeutical effects

of a *Serenoa repens* extract Me180 versus tamsulosin

in patients with obstructive benign prostatic hyperplasia

1 Introduction and aim of the pilot study

Several plant extracts are widely used for the treatment of symptomatic benign prostatic hyperplasia (BPH) (Table II-1). Despite their traditional popularity, the role of phytotherapeutic agents for this treatment is continuously debated. Clinical studies have mainly assessed the effectiveness of *Serenoa repens* extracts (SRE). In a recent meta-analysis of controlled trials Wilt and co-workers noted that the effect of SRE significantly exceeded that of placebo with regard to overall urinary symptoms, nocturia and the maximal urinary flow rate (Wilt et al 2002a). However, in most of these trials, a number of shortcomings were identified, including a lack of rigid inclusion and/or exclusion criteria defining the patient population, short duration of the study, failure to administer a validated symptom questionnaire and including too few subjects. There is a lack of studies planned and conducted under strict methodology. In many studies, no placebo effect was noted, raising additional concern since placebo mediated improvement was noted in virtually all clinical trials of BPH pharmaceuticals. Moreover, conclusions of meta-analysis that include products from different plants undergoing different extraction procedures may be misleading.

Two large, randomized, double blind studies have shown a *Serenoa repens* hexane extract (Permixon®) to be as effective as the uro-selective α-adrenergic blocker, tamsulosin, and the 5α-reductase inhibitor, finasteride, in achieving subjective and objective improvements of symptomatic BPH (Carraro et al 1996; Debruyne et al 2002). Thus, the available data may suggest but do not clearly establish the efficacy and safety of the SRE for symptomatic BPH.

Moreover, the most widely studied form of SRE is Permixon®, which uses the solvent hexane, other formulations have used ethanol, and liquid carbon dioxide as solvent. In contrast to the well studied Permixon®, our study medication consists of the hydroethanolic (96%) *Serenoa repens* extract Me180 (SabCaps®). SabCaps® is registered by the Swissmedic (No. 54823) and sold as over-the-counter in Switzerland since 2001, without any previous conducted clinical studies. Direct comparison between different products is impossible because of the different extraction procedures used by the different manufacturers, the variations of the raw plant material used and the fact that the potentially active component(s) of the final

product and its (their) mechanism(s) of action are still under investigation. Thus, one product might show clinical efficacy while another might not. Therefore, the results of basic research and clinical trails cannot be transferred from one manufacturer's product to another, and each manufacturer's preparation must be evaluated separately, using the gold standard of placebo-controlled studies or at least reference-controlled studies according to accepted guidelines.

A major problem associated specifically with the objective evaluation of new therapies for BPH is the lack of consensus over the definition of BPH. This controversy is related principally to the terminology and how these terms are interpreted with resultant consequences for both the diagnosis of patients and the subsequent evaluation and follow up. The use of a definition narrows the concept of BPH by delineating a 'cleaner' patient population. This would have an impact on clinical management and yield more trustworthy research results. Unfortunately, most studies on incidence and prevalence of BPH do not encompass the same type of patients, because the definition of BPH is often lacking or is based on symptoms, or prostatic volume, or obstruction in isolation (Hald et al 1998). Therefore, we decided to narrow the patient collective to patients with obstructive BPH.

The selective $\alpha 1_A$-blocker, tamsulosin, can be considered as the first-line treatment option for symptomatic BPH because it provides effective relief of bothersome lower urinary tract symptoms (LUTS) with excellent tolerability (Schulman 2003). Due to the lack of any clinical investigations with the *Serenoa repens* extract Me180, the present pilot study was performed to systematically assess the therapeutical effects, safety and tolerability of the *Serenoa repens* extract Me180 versus tamsulosin as an active control in patients with obstructive BPH treated for 26 weeks. Primary and secondary outcome criteria were assessed according to the recommendations of the 4th International consensus committee on BPH (Roehrborn 1998).

The aim of this pilot study is the systematical assessment of therapeutical effects, safety and tolerability of a 26 week-treatment with *Serenoa repens* fruit-extract Me180 (320mg/d) (SabCaps®) compared to tamsulosin (0.4mg/d) (Pradif®) in patients with obstructive BPH. The outcome of the present study was intended to form the basis for a subsequent, extended study with more patients to assess the efficacy of the extract over a longer treatment period.

2 Synopsis

Objectives:	Assessment of the efficacy, safety and tolerability of a *Serenoa repens* fruit-extract Me180 (320mg/d) versus tamsulosin (0.4mg/d) as an active control for patients with obstructive BPH
Study design:	Pilot study: prospective, randomised, double-blind, tamsulosin-controlled, parallel-group comparison over 26 weeks
Types of participants:	19 patients with obstructive benign prostatic hyperplasia
Inclusion criteria:	• Age: ≥ 45 years ≤90 years • International Prostate Symptom Score IPSS > 6 • Averaged peak urinary flow rate Q_{max} < 10ml/s for a voided volume of at least 150ml assessed twice at two different days, if averaged Q_{max} 10-15ml/s obstruction has to be ascertained by pressure-flow study • Postvoid residual urine volume ≤200ml • Serum prostate-specific antigen (PSA) <10ng/ml for prostates ≤ 60ml <15ng/ml for prostates > 60ml > 3ng/ml biopsy for exclusion of prostate cancer • Participants were required to declare their informed consent to participate in writing. • No indications for any acute medical treatment or for operative intervention.
Exclusion criteria	• Co-medication that might interfere with study medication, including hormone preparation, cimetidine, cyclosporine A, anticoagulants, diuretics, calcium channel blockers, antidepressants and neuroleptics, anticholinergics, sympathomimetics, parasympatholytics, 5α-reductase inhibitors, or any other phytotherapeuticals intended for BPH-treatment. If such drugs had been used in the past 3 months, patients had to be excluded from the study. • Diabetes • Cardiovascular disorders • Hypertension • Orthostatic hypotension • Lower urinary tract infection • Retention of urine, hydronephrosis, renal insufficiency • Previous urological surgery (including the prostate, bladder and urethra) • Prostate cancer • marked disorders of other organ systems including renal or hepatic insufficiency • known pathological laboratory values (blood: abnormal ASAT, ALAT, alkaline phosphatase, creatinine > 150μmol/l; urine: leucocyte count and urine protein > 30mg/dl) • Senile dementia • Alcohol or drug abuse • Known history of drug hypersensitivity • Physical findings suggesting surgery in near future • Participation in another trial within the past 3 months. • Diseases affecting absorption such as crohn's disease and celiac disease
Study medication:	***Serenoa repens* fruit extract Me180** (320mg hydroethanolic extract (96%),SabCaps®) or matching **tamsulosin HCl** (Pradif®) 400μg one capsule daily

Treatment duration:	26 weeks
Outcome measures:	Primary outcome measures • Peak urinary flow rate • I-PSS (Internationale Prostate Symptom Score) with quality-of-life index QoL Secondary outcome measures • Mean urinary flow rate • Residual urine volume • Prostate size • IIEF (Internationale Index of Erectile Function)
Safety assessment:	• Adverse effects • pulse and blood pressure (after sitting 5' and subsequently standing up) and routine laboratory tests were assessed (serum values of alkaline phosphatase, aspartate aminotransferase ASAT, alanine aminotransferase ALAT, haemoglobin, creatinine, and prostate specific antigen (PSA) and urinalysis for leukocyte count and urine protein)
Settings:	Department of Urology, University Hospital, in Basel and Liestal, Switzerland.
Visits	pretrial selection, controls at weeks 0, 2, 8, 16, 26

Table IV- 1 Monitoring schedule:

week	**-1**	**0**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**	**24**	**26**
visit no.	**I**	**II**	**III**			**IV**				**V**					**VI**
	selection	begin	control 1			control 2				control 3					termination
treatment		———	———	———	———	———	———	———	———	———	———	———	———	———	———
blood pressure and pulse	x		x			x				x					x
blood and urine withdrawal	x														x
IPSS, quality-of-life index	x		x			x				x					x
peak urinary flow rates	x	x	x			x				x					x
mean urinary flow rates	x		x			x				x					x
postvoid residual urine	x		x			x				x					x
IIEF	x					x				x					x
prostatic volume	x														x

3 Patients and Methods

3.1 Design

This prospective, double-blind, randomised, active-controlled, parallel-group, pilot study was conducted at the Department of Urology, University Hospital, in Basel and Liestal, Switzerland. The pilot study was a 26 week, two-arm comparison, to systematically assess the therapeutical effects, safety and tolerability of the *Serenoa repens* extract Me180 (SabCaps®) versus tamsulosin (Pradif®) as an active control in patients with obstructive BPH treated for 26 weeks. Figure IV-1 shows the study design.

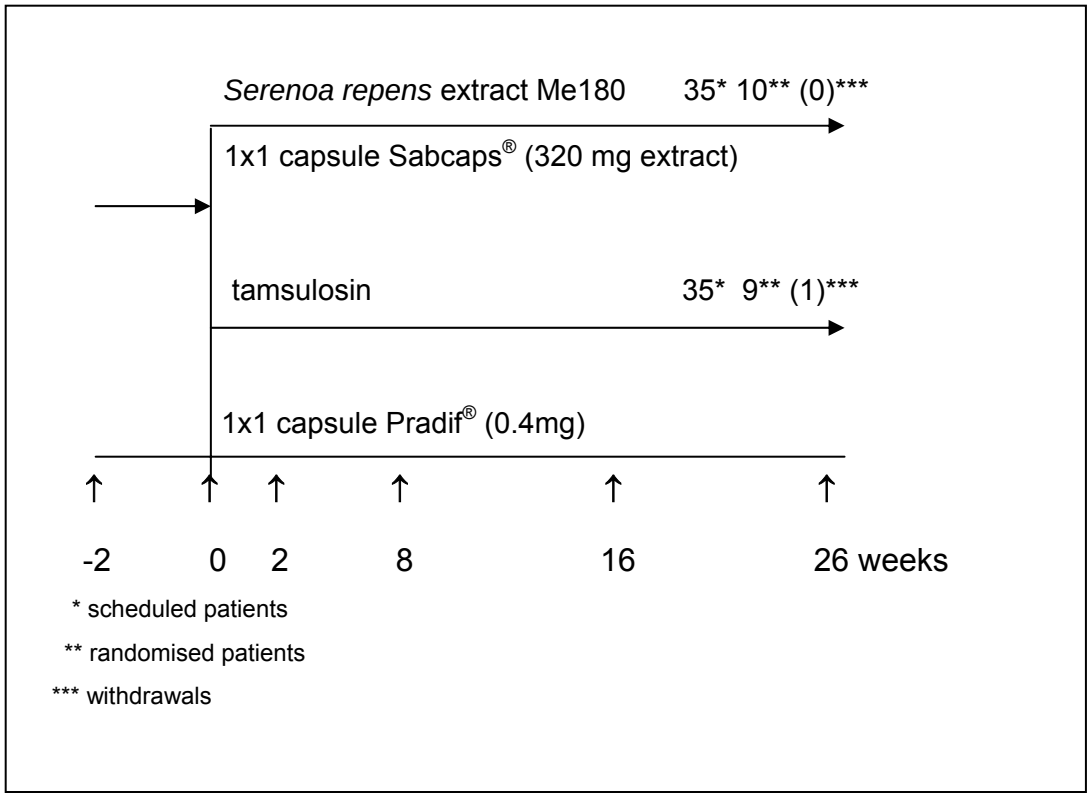

Figure IV- 1 Study design

According to the calculations (3.8.1 required sample size) 70 patients were scheduled to be included in the pilot study, 35 for each treatment group. Patient recruitment was stopped prematurely, thus, just 19 patients with obstructive BPH were included in the double-blind phase of the study, of which 10 received 320mg *Serenoa repens* extract Me180 and 9 received 0.4mg tamsulosin once daily in the morning. The main part of the trial encompassed 26 weeks of randomised treatment

during which there were four further visits, after 2,8,16 and 26 weeks. Patients were randomised at start of treatment (week 0) into two study groups.

3.2 Study population and selection of patients

3.2.1 Inclusion and exclusion criteria

Men, aged 45 to 90 years, were **included** in the trial if they had moderate BPH according to their International Prostate Symptom Score (I-PSS) (> 6 points) and an averaged peak urinary flow (Q_{max}) of < 10ml/s for a voided volume of 150ml measured at visit -2 and visit week 0. If Q_{max} was 10-15ml/s obstructive BPH had to be ascertained by pressure-flow study. Other inclusion criteria were a residual urine volume of ≤ 200ml, prostate specific antigen (PSA) < 10ng/ml for prostate ≤ 60ml, <15ng/ml for prostate> 60ml and no indications for any acute medical treatment nor for operative intervention. Participants were required to declare their informed consent to participate in writing.

Exclusion criteria were concomitant medication likely to interfere with study medication, including hormone preparation, cimetidine, cyclosporine A, anticoagulants, diuretics, calcium channel blockers, antidepressants and neuroleptics, anticholinergics, sympathomimetics, parasympatolytics, 5α-reductase inhibitors, or any other phytotherapeuticals intended for BPH-treatment. If such drugs had been used in the past 3 months, patients had to be excluded from the study. Furthermore, patients with any of the following disorders were also excluded: presence of urinary tract infections, diabetes, cardiovascular disorders, hypertension, orthostatic hypotension, retention of urine, hydronephrosis, renal insufficiency, previous urological surgery (including the prostate, bladder and urethra), prostate cancer, severe organic diseases, marked disorders of other organ systems including renal or hepatic insufficiency, senile dementia, alcohol or drug abuse, known history of drug hypersensitivity, physical findings suggesting surgery in near future, diseases affecting absorption such as crohn's disease and celiac disease, or participation in another trial within the past 3 months. Patients who fulfilled one of these exclusion criteria were not allowed to participate in the study.

3.3 Recruitment and patient selection

Patients were recruited by several local newspapers of Basel and environments. Participants were screened for eligibility by telephone interview, and at the first visit, and they were randomised at the second one. Men who had severe symptoms like increased frequency of urination, hesitancy and poor stream, were invited to the first visit. They were required to be aged between 45 and 90 years and to have no items mentioned under exclusion criteria (3.2.1 Inclusion and exclusion criteria). At the first visit, patients with obstructive BPH were enrolled into the study (Figure IV-2).

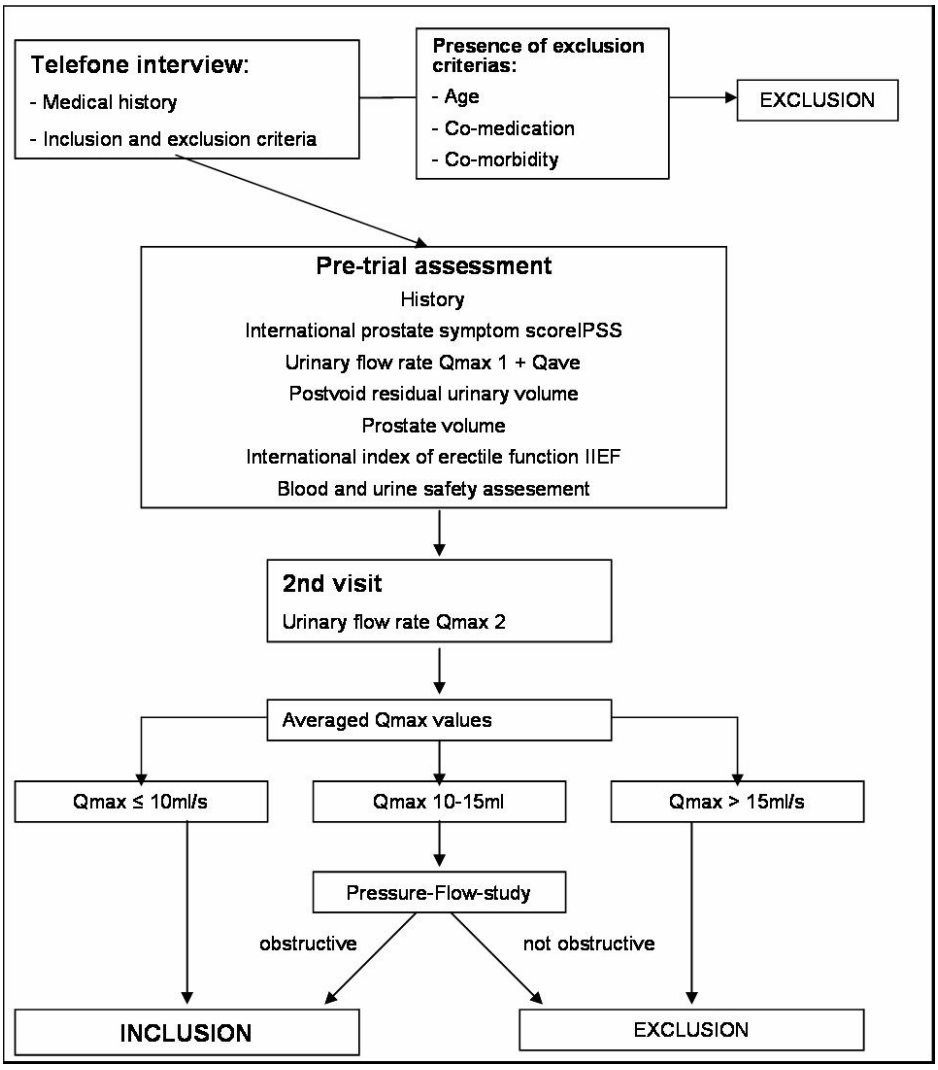

Figure IV- 2 Pilot study profile

3.4 Examination procedures

The 4th International Consensus on BPH recommended a duration of at least 12 months for clinical trials to document the efficacy for BPH treatment. We decided to conduct the first pilot study with a duration of 6 months. The study consisted of six hospital visits. The main part of the trial encompassed 26 weeks of randomised treatment during which there were 4 further visits, at week 2,8,16 and 26. Subjective and objective outcome assessments were carried out according to the monitoring schedule outlined in Table IV-1. The time flow of the visits was adhered to with accepted deviations of ±1 week. Because of circadian changes of uroflowmetry (Golomb et al 1992) and postvoid residual urine volume (Griffith et al 1996) all assessments were carried out in the morning.

At visit I (pre-trial assessment), medical history was taken to identify other causes of voiding dysfunction, symptoms were evaluated using the International Prostate Symptom Score (IPSS), and quality-of-life (QOL), peak (Qmax) and mean (Qave) urinary flow rate, postvoid residual urine, prostate volume, pulse and blood pressure (BP), after sitting 5' and subsequently standing up, and routine laboratory tests were assessed. Patients who fulfilled the inclusion criteria were invited for visit II, if they wished to participate.

Biopsy of the prostate was proposed to patients with elevated PSA values (>3ng/ml) according to the reference ranges used locally. If the biopsies were negative for carcinoma, the patient was invited for the second visit.

At visit II, Qmax was assessed and averaged with Qmax of visit I giving the baseline Qmax. Patients with baseline Qmax < 10ml/s were included, if averaged Qmax 10-15ml/s, the obstruction had to be ascertained by pressure-flow study.

Visits III,IV,V and VI were scheduled 2,8,16,and 26 weeks later. At each visit, the patients were asked to fill out the IPSS questionnaire and objective measurements peak (Q_{max}) and average (Q_{ave}) urinary flow rates, the residual urine volume, systolic and diastolic blood pressure and pulse rate were assessed. At visits IV, V and VI patients were asked to fill out the IIEF questionnaire. Patients were interviewed regarding concomitant medication and occurrence of adverse events. Information of adverse events was collected based on a standardized questionnaire according to good clinical practice guidelines, which included type of adverse events, beginning

and duration, intensity, frequency, necessity for adverse event treatment, assumed relationship with study medication, severity and outcome. At visit VI (study termination), additionally, prostate volume was assessed and physicians and patients were asked a global subjective question to estimate the efficacy of the treatment on a five-stage scale and physicians were asked a second question concerning the tolerability.

3.5 Outcome and efficacy parameters

The efficacy of the two study treatments was assessed according to the recommendations of 4th International Consultation on BPH (1997) (Roehrborn 1998).

3.5.1 International Prostate Symptom Score (IPSS)

The most widely used symptom score is the American Urological Association Symptom Score (AUA-SS) and with the addition of a single disease specific question on quality-of-life (added by the WHO) becomes the International Prostate Symptom Score **IPSS** (Barry et al 1992) (Table IV-2). Unlike previously published symptom scores, this index has been designed for self-administration in a uniform manner. A validated German version of the IPSS was filled out by the patients.

Table IV- 2 International Prostate Symptom Score IPSS made up of the American Urological Association Symptom Score (Barry et al 1992) and a single question to assess quality-of-life added by WHO.

Question	Not at all	Less than one time in five	Less than half the time	About half the time	More than half the time	Almost always
Incomplete emptying						
Over the last month, how often have you had a sensation of not emptying your bladder completely after you finish urinating?	0	1	2	3	4	5
Frequency						
Over the last month, how often have you had to urinate again less than two hours after you had finished urinating?	0	1	2	3	4	5
Intermittency						
Over the past month, how often have you found you stopped and started again several times when you urinated?	0	1	2	3	4	5
Urgency						
Over the past month, how often have you found it difficult to postpone urination?	0	1	2	3	4	5
Weak Stream						
Over the past month, how often have you had a weak urinary stream?	0	1	2	3	4	5
Straining						
Over the past month, how often have had to push or strain to begin urination?	0	1	2	3	4	5
Nocturia						
Over the past month, how many times did you most typically get up to urinate from the time you went to bed at night until the time you got up in the morning?	0	1	2	3	4	5
Total AUA-SS						

Quality of life due to urinary symptoms	**Delighted**	**Pleased**	**Mostly satisfied**	**Mixed-about equally satisfied and dissatisfied**	**Mostly dissatisfied**	**Unhappy**	**Terrible**
If you were to spend the rest of your life with your urinary condition just the way it is now, how would you feel about that?	0	1	2	3	4	5	6

The IPSS comprises a set of seven questions on urinary function, covering frequency, nocturia, weak urinary stream, hesitancy, intermittence, incomplete emptying and urgency. Each question is measured on a 5-point scale, resulting in a total score of 35 points. 'Obstructive' symptoms are assessed by questions 1, 3, 5 and 6 (total 20 points), while 'irritative symptoms' are assessed by questions 2, 4, and 7 (total 15 points).

In recognition of the importance of the disease specific quality-of-life, and the degree to which it is affected by the symptoms, a single quality-of-life question was added to the symptom score (Table IV-2). This answer is rated on a 0-6 point scale, where the highest score corresponded to the worst perception.

3.5.2 Urinary flow rate

Urinary flow rate recording is a non-invasive technique to assess the strength of the urinary stream using an uroflowmeter.

The peak and average urine flow rates are those rates most usefully.

Peak urinary flow rate: Q_{max}, greatest flow per unit of time

Average flow rate: Q_{ave}, the voided volume divided by the flow time

Presently, the only available direct method to assess the presence or absence of obstruction is a pressure-flow study. Urine flow studies are limited by their failure to identify detrusor underactivity as a cause of low flow rates and by their failure to indicate obstruction in those patients who have normal flow rates but ‚supranormal' detrusor voiding pressures. However, this non-invasive method has gained wide-spread use to assess obstruction. It was shown that 90% of patients with peak flow rate of less than 10ml/s have bladder outlet obstruction (BOO) while this percentage decreased to 67% in those with a Q_{max} of 10-15ml/s. The specificity will depend on the patient group, but two series correlated well. The following data for 3 bands or Q_{max} gives the percentages of patients who will be obstructed (Abrams 1998).

Qmax < 10ml/s	90% BOO
Qmax 10-14ml/s	67% BOO
Qmax > 15ml/s	30% BOO

As urinary flow rates are dependent on voided volume, a voided volume of > 150ml is required for an accurate reading (Haylen et al 1989). Controversy exists regarding the reproducibility of this parameter in the same individual on repeated measurements (Golomb et al 1992). A single flow rate does not give adequate information, except when it is clearly normal (e.g. Q max >25ml/s) and has a bell-shaped course over time. Therefore, patients were only included with an averaged peak flow rate of < 10ml/s measured on two different days (Abrams 1998). In patients with Q_{max} 10-15ml/s obstruction had to be ascertained by pressure - flow study.

3.5.3 Postvoid residual urine volume

The amount of urine left in the bladder after urination was measured by transabdominal ultrasonography. Healthy volunteers have been found to have less than 12ml of residual urine (Roehrborn 1998). Thus, the presence of residual urine constitutes an abnormal finding, although it is certainly not specific for the diagnosis of BPH. While originally designed with a minimum of 30ml < R < 200ml, during the study, this criterion needed to be adjusted to R< 200ml because of the loss of patients.

3.5.4 Prostate size

It is a well established fact that prostate size correlates only moderately with patients' symptoms (Roehrborn 1998). However, prostatic enlargement is one of the three aspects determining the picture of clinical BPH (Figure II-3). Therefore, prostatic size was assessed as a secondary outcome by transabdominal ultrasonography at baseline, and after 26 weeks of treatment.

3.5.5 International Index of Erectile Function IIEF

Diseases of the prostate may interfere with sexual function and treatment of prostatic diseases may exhibit an impact on sexuality (Burger et al 1999). To assess the effect of the treatments on sexual function the International Index of Erectile Function (IIEF) was completed. IIEF is a 15-item, self-administered questionnaire scale developed for the assessment of sexual function (Rosen et al 1997). Five factors or response domains were identified: erectile function, orgasmic function, sexual desire, intercourse satisfaction, and overall satisfaction.

3.5.6 Urodynamic pressure-flow studies

Pressure flow studies are invasive studies that simultaneously measure urinary flow rates, bladder pressure, and abdominal pressure. To avoid the discomfort and potential complications, obstructive BPH was only diagnosed by pressure flow studies, in patients with flow rates between 10-15ml/s.

3.5.7 Global subjective assessment of the treatment outcome and its tolerability

Additionally, a global subjective assessment of the efficacy was added by an overall question to patients and physicians. The five-stage answer scheme ranges from ”very good” to ”none”.

3.6 Safety assessment and documentation of adverse effects

The safety of the study medication was evaluated by laboratory monitoring at baseline and endpoint of serum values of alkaline phosphatase, aspartate aminotransferase ASAT, alanine aminotransferase ALAT, haemoglobin, creatinine, and prostate specific antigen (PSA) and urinalysis for leukocyte count and urine protein. All the analyses were performed at the laboratory of the University Hospital in Basel.

At every visit pulse and blood pressure were assessed after sitting 5’ and standing up.

In every consultation the participants were asked if they had noticed any adverse effect and mentioned effects were documented based on a standardized questionnaire according to good clinical practice guidelines, which included type of adverse events, beginning and duration, intensity, frequency, necessity for adverse events treatment, assumed relationship with study medication, severity and outcome.

After completing the therapy, physicians were asked a global subjective question to estimate the tolerability of the treatment on a five-stage scale, ranging from “very good” to “very poor”.

If the physicians estimated the tolerability with “moderate”, “poor” or “very poor” they were asked to further explain.

3.7 Study medication

Study medication consisted of either *Serenoa repens* fruit extract Me180 320mg/day (SabCaps®, 96% ethanol m/m, extract ratio 9-11:1) or the reference preparation of tamsulosin 0.4mg/day (Pradif®). Both drugs are commercially available and admitted by the Swissmedic, SabCaps® no. 54823 and Pradif® no. 53471. The first group received 320mg of *Serenoa repens* fruit extract Me180 (SabCaps®) per day and the second group received 0.4mg tamsulosin (Pradif®) per day, both in the morning. The used dosages of both regimens were based on the recommendations of the suppliers. Treatment was given for a total of 26 weeks.

3.7.1 Blinding

The drugs were blinded with special gelatine capsules for double-blind clinical trials DBcaps® supplied by Capsugel AG, Bornem, Belgium, and blinding of medication was conducted by Fisher Clinical Services, Pharmaceutical Packaging, Horsham, England, and blind-packaging by Fisher Clinical Services, Pharmaceutical Packaging, Allschwil, Switzerland. We used a single product lot throughout the study, and preparations were matching for appearance, size, colour, taste, and smell.

3.7.2 Randomisation

The patients were allocated symmetrically to the two treatment groups according to a randomisation schedule created by the statistician with random block sizes of four. The patient numbers were assigned to the patients chronologically in ascending order. Randomisation codes were concealed in sealed envelopes and opened only after the last man had completed treatment.

3.7.3 Compliance

Patients were informed at first and at all following visits about the importance of the conformable intake of the study medication. Patient compliance was estimated from capsule counts at each visit. Average compliance, assessed by counting the remaining capsules at each visit, was 98% for all men during the trial. No subject had a compliance of less than 93%.

3.8 Co-medication

During the study, every co-medication which is not itemised under exclusion criteria was allowed. However, all co-medication had to be registered on a special form.

3.9 Biometrical planning and analysis

3.9.1 Required sample sizes

Based on results of conducted trails (Wilt et al 1998; Wilt et al 2002b) the following assumption was made: the mean difference of improvement for the peak urinary flow rate between *Serenoa repens* extract and tamsulosin is δ=0.88 (detectable treatment difference), for the improvement the pooled standard deviation is σ=1.17 (compare with (Carraro et al 1996)). Based on these specifications, the significance level α=0.05, the power 1-β=0.80 and the power determination by means of t-test the required sample sizes are 28 patients by treatment group (calculated with software Sample Power (Version 1.2), SPSS inc., 1997). Taking into consideration a dropout rate of 25% 35 patients should be included in each treatment group.

3.9.2 Statistical methods

The statistical evaluation was done based on the “Per-Protocol (PP)” principle. The two-sided alternative hypothesis was stated. The paired differences “under treatment minus before treatment” were the basic data to compare the two treatment efficacies. The parameters were described with usual statistical methods in an overall analysis according to the PP principle.

- *t-test* for comparing the means of two independent samples under parametric normality condition.
- *Paired t-test* for comparing the means of two paired samples under parametric normality condition.
- *Kruskal-Wallis test* for comparing the means of two independent samples under non-parametric condition.
- *Wilcoxon test* for comparing the means of two paired samples under non-parametric condition.
- Chi square test for analyzing contingency tables
- Fisher’s exact test for analyzing contingency tables

The significance level α=0.05 is used in statistical test applications. The two-sided probability p is determined according to the stated two-sided alternative hypothesis.

The probability p can shortly be interpreted as follows:

	$p > 0.10$:	no statistical significance
$0.05 <$	$p \leq 0.10$:	tendency of statistical significance
$0.01 <$	$p \leq 0.05$:	slight statistical significance
$0.001 <$	$p \leq 0.01$:	statistical significance
	$p \leq 0.001$:	high statistical significance

The statistical software tools, Microsoft EXCEL, Version 7.0 (1997) and SAS/STAT Version V8.02. (1999) were mainly used to describe and evaluate data collected in the present pilot study *Serenoa repens* extract versus tamsulosin.

3.9.3 Data recording and statistical evaluation

Data were acquired once on a paper version. In addition, an electronic case report form (eCRF) developed by pnn, pharma nation network AG, Zürich, was included to test electronic data entry. All data were analysed by Consult AG, Dr. U. Kreuter, Bern- Liebefeld.

3.10 Ethical and legal considerations

The study preparation, execution, and analysis were performed according to current European Union and International Conference on Harmonisation guidelines on Good Clinical Practice and the Declaration of Helsinki with revision of Edinburgh, Scotland, October 2000, and the regulations of Swissmedic (Reglement über die Heilmittel im klinischen Versuch).

The study was approved by the ethics committee of Basel and notified to Swissmedic.

Written informed consent was obtained from all participating patients before treatment.

Personal data obtained during the study are protected and were not given to any unauthorised person.

Participations were insured by the ‘Probandenversicherung des Kantons Basel-Stadt’.

4 RESULTS

4.1 Patients distribution

Between August 2002 and March 2003, 420 men living in Basel and region answered to the 6 advertisements placed in several local newspapers. Participants were screened for eligibility by a telephone interview, and 108 patients were invited for the first visit (Figure IV-3). 312 patients were excluded due to exclusion criteria, particularly because of medication possibly interfering with the study medication (Table VI-3). Of the 108 men invited to attend the initial visit for history and diagnosis of obstructive BPH, 19 were eligible to be randomised, 10 to the *Serenoa repens* Me180 arm and 9 to the tamsulosin arm (Figure IV-3). Of these, 10 of the Me180 and 8 patients of the tamsulosin group completed the study. Only 1 patient of the tamsulosin group discontinued the study because of continuous dizziness.

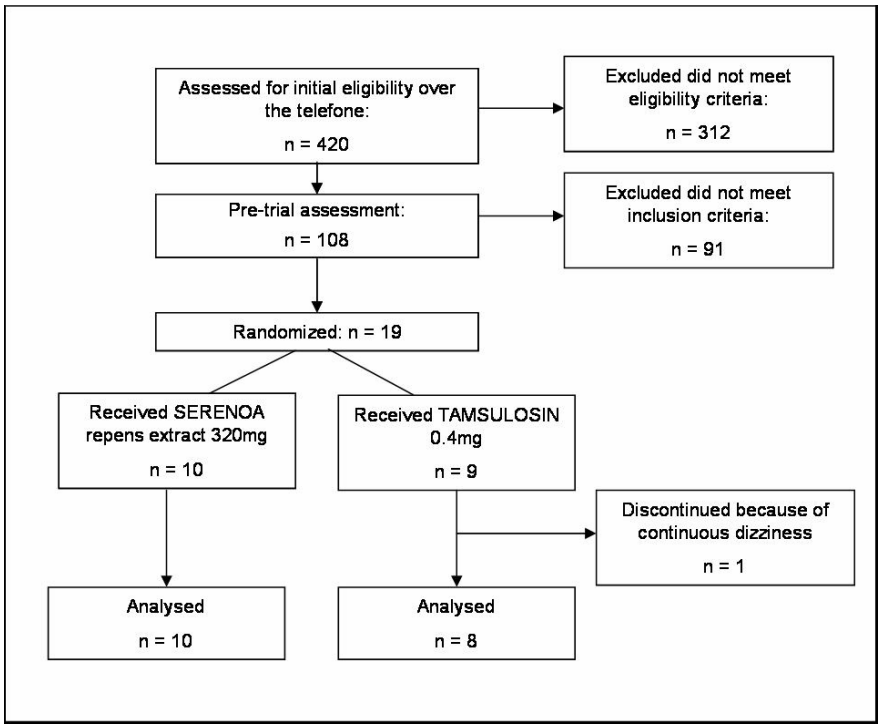

Figure IV- 3 Trial diagram showing the distribution of participants at each stage.

The patient recruitment was stopped because of a high dropout rate at the telephone interview (74%) (Table IV-3), but also at the first visit (82%) (Table IV-4), so that only 4.5% of interested participants were finally included and randomised in the pilot study.

Cardiovascular medication and pre-existing BPH treatment were the most frequent reasons why patients could not be included after the telephone interview (Table IV-3).

Table IV- 3 Excluded at the telephone interview

Exclusion criteria at telephone interview	No of patients	in % within	% overall
interfering medication	205	65.7	48.8
too young	20	6.4	4.8
wished phytotherapeutic medication	6	1.9	1.4
high franchise or HMO insurred	13	4.2	3.1
no disorders	25	8.0	6.0
unable to understand the significance of the trial	3	1.0	0.7
miscellaneous	40	12.8	9.5
Total initially excluded patients	312	100.0	74.3

After the first visit, 89 patients were not eligible for the inclusion in the study, mainly because the flow rate was> 15ml/s or > 10ml/s and they were not willing to have a pressure-flow study done (Table IV-4). In 3 included patients obstructive BPH was assessed by pressure-flow study.

Table IV- 4 Excluded at the first visit

Exclusion criteria at the first visit	No of patients	in % within
Peak urinary flow	43	39.8
Residual urine volume	17 (3)*	15.7
PCA	4	3.7
Miscellaneous	15	13.9
Total initially excluded patients	89	82.4

*At the beginning of the pilot study, there was a minimum of 30ml residual urine volume required. These restrictions where eased after loss of patients. After protocol change there were 3 patients with R > 200ml, who needed to be treated beyond the study.

4.2 Baseline characteristics

The randomised groups were similar in their baseline characteristics as shown in Table IV- 5. There were no significant differences in the characteristics (p>0.1). The mean age of patients in the Me180 group was 62.8 (50-74) years, while the mean age of the tamsulosin group was 61.4 (52-79) years.

Table IV- 5: Baseline patient characteristics at selection visit

Characteristics	**Me180 (n=10)**	**tamsulosin (n=8)**	
No. randomised	10	9	
No. completing study	10	8	
	Me180 (n=10)	tamsulosin (n=8)	p value
Duration of symptoms	2.47 ± 1.23	2.61 ± 1.67	0.77
Age (years)	62.8 ± 8.2	61.4 ± 9.1	0.73
Body mass index[a]	25.40 ± 2.5	25.42 ± 2.13	0.98
IPSS (0-35 points)	16.9 ± 5.74	19.13 ± 6.27	0.48
QoL (0-6 points)	2.1 ± 1.45	2.5 ± 1.6	0.55
Peak urinary flow rate Qmax	9.17 ± 2.80	7.78 ± 2.34	0.35
Mean urinary flow rate Qave	5.35 ± 2.09	4.59 ± 1.33	0.33
Postvoid residual urine volume (ml)	58.9 ± 70.82	26.0 ± 17.20	0.42
Prostate volume (ml)	45.9 ± 22.18	39.0 ± 14.66	0.72
Serum PSA (ng/ml)	1.83 ± 1.53	1.16 ± 0.53	0.65
[a] kg/m^2 Values are means ±SD			

4.3 Treatment outcome

Table IV-6 shows the evolution of IPSS, the quality-of-life index, the maximum and mean flow rates, the residual urinary volume and the prostate volume over the 26 weeks treatment period. For these parameters, the effects in the two treatment groups were not significantly ($p>0.1$) different, meaning that the evolution was comparable in both groups. In detail, the single evolutions are shown in the following chapters.

Table IV- 6: Study results[a] from baseline to endpoint

Parameter	Group	n	Baseline	week 2	week 8	week 16	endpoint
IPSS	Me180	10	16.9 ± 5.7	16.1 ± 6.9	13.9 ± 6.7	13.6 ± 5.4	13.1 ± 7.6
(points)	tamsulosin	8	19.1 ± 6.2	16.6 ± 3.0	15.8 ± 2.3	14.0 ± 4.2	14.8 ± 4.8
QoL	Me180	10	2.1 ± 1.5	3.4 ± 1.6	2.4 ± 1.4	2.7 ± 1.3	2.0 ± 1.2
(points)	tamsulosin	8	2.5 ± 1.6	2.6 ± 1.2	2.4 ± 0.9	2.1 ± 1.1	2.0 ± 1.1
Q_{max}	Me180	10	9.2 ± 2.8	11.8 ± 5.5	12.4 ± 4.3	10.8 ± 5.4	10.1 ± 5.3
(ml/s)	tamsulosin	8	7.8 ± 2.3	9.9 ± 4.4	12.0 ± 5.5	10.8 ± 5.2	11.8 ± 8.8
Q_{ave}	Me180	10	5.4 ± 2.1	6.6 ± 3.5	6.4 ± 1.9	5.7 ± 2.4	5.0 ± .2
(ml/s)	tamsulosin	8	4.6 ± 1.3	5.0 ± 2.3	5.6 ± 2.0	5.7 ± 2	4.6 ± 1.2
R	Me180	10	58.9 ± 70.8	56.3 ± 53.0	54.8 ± 46.8	52.6 ± 58.8	53.2 ± 31.1
(ml)	tamsulosin	8	26.0 ± 17.2	37.1 ± 43.5	57.1 ± 49.9	26.8 ± 25.9	43.4 ± 43.9
P	Me180	10	45.9 ± 22.2	n.d.	n.d.	n.d.	47.5 ± 18.2
(ml)	tamsulosin	8	39.0 ± 14.6	n.d.	n.d.	n.d.	38.7± 8.4

a Data are presented as mean (± standard error (s.e.))

n.d. not determined

4.4 Primary outcome measures

4.4.1 International Prostate Symptom Score (IPSS)

After 26 weeks both treatments decreased the total IPSS in a similar pattern and to a similar extent with -3.8 points in the Me180 group and -4.38 points in the tamsulosin group. Comparing the means and means of paired differences “26 minus 0 weeks” no statistical significance was demonstrated between both treatment groups (p= 0.48). Figure IV-4 shows the evolution of the total IPSS.

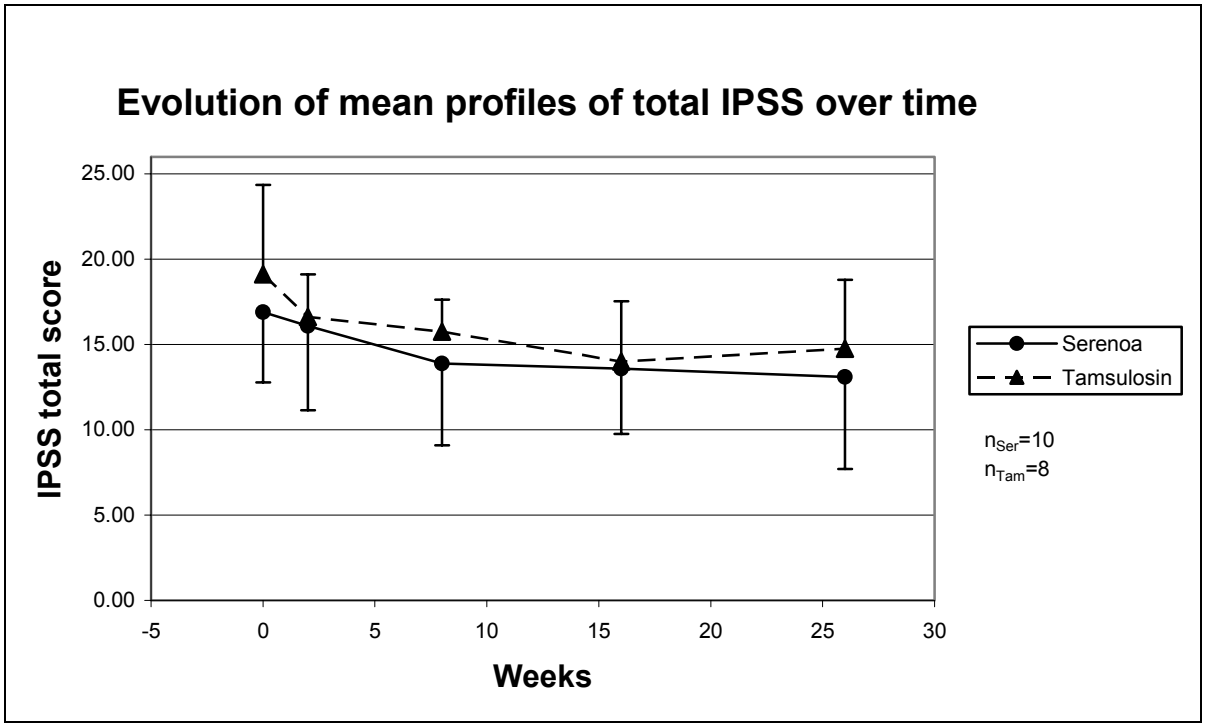

Figure IV- 4 Evolution of total IPSS means with 95% confidence intervals (CI) of Me180 and Tamsulosin treatment groups at five control time-points (weeks 0, 2, 8, 16, 26).

At baseline, the score was slightly greater in the tamsulosin group but not statistically significant (p=0.48). At week 16, the decrease with respect to baseline in both treatment groups was statistically slightly significant (p = 0.03) for Me180 and with tendency of significance (p= 0.10) for the tamsulosin group. For both treatments, the mean percentage change from baseline to endpoint (26 weeks) was similar (22.5% for Me180 and 22.9% for tamsulosin).

Non significant differences were observed between the treatment groups at 26 weeks in terms of irritative symptoms (p=0.93) and obstructive symptoms (p=0.53) (Figure IV-5 and IV-6). However, within the treatment groups, Me180 showed a slight significance in the decrease in irritative symptoms (p=0.03) and a tendency to significance in the decrease of obstructive symptoms in both treatment groups (p=0.07).

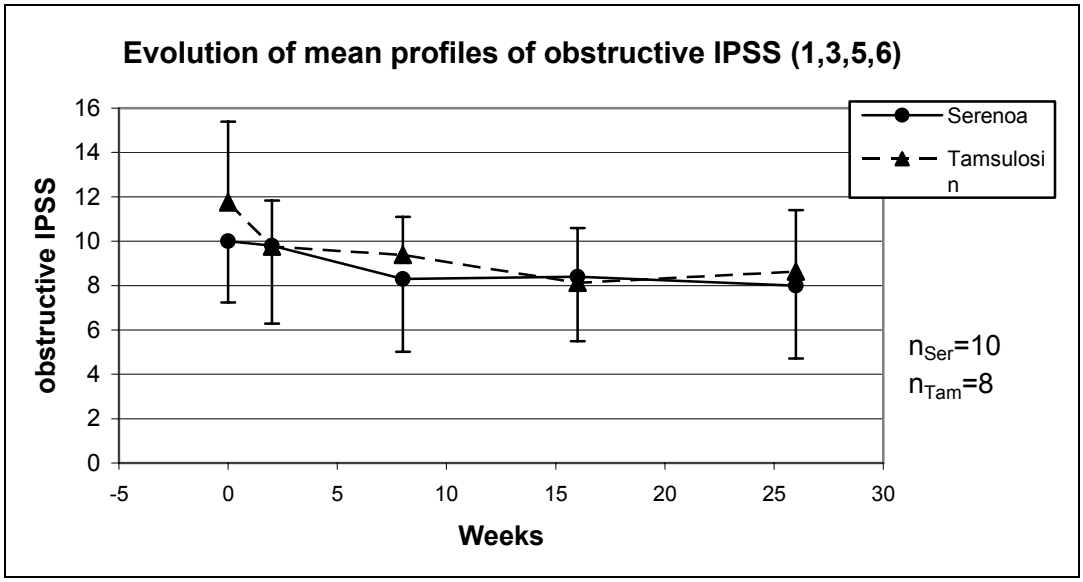

Figure IV- 5 Evolution of obstructive symptom score (IPSS 1,3,5,6) means with 95% CI of Me180 and tamsulosin treatment groups at five control time-points (weeks 0, 2, 8, 16, 26).

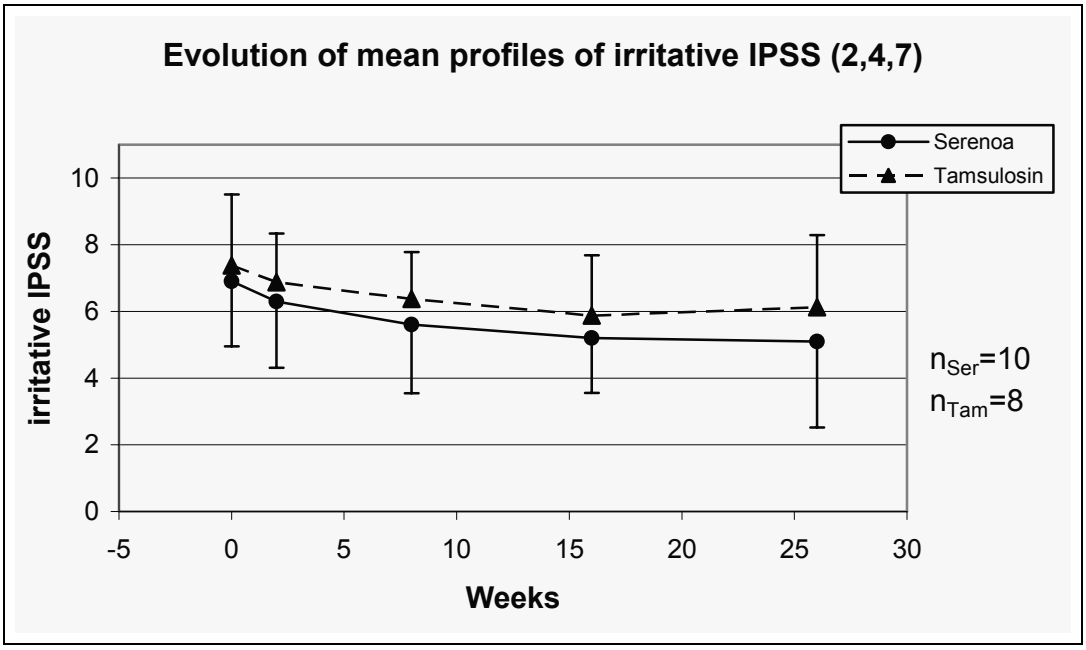

Figure IV- 6 Evolution of irritative symptom score (IPSS 2,4,7) means with 95% CI of Me180 and tamsulosin treatment groups at five control time-points (weeks 0, 2, 8, 16, 26).

Furthermore, the analysis of each of the IPSS items showed no significant difference between the two preparations (Figure IV-7). Table IV-7 shows the evolution of the means over the 26 weeks with corresponding p values within the treatment groups and between the treatment groups. The strongest decrease of score was found for weak stream (IPSS-5), incomplete emptying (IPSS-1) and nocturia (IPSS-7).

Table IV- 7 Means for individual IPSS questions (1-7) of treatment groups Me180 and tamsulosin in 5 controls (week 0,2,8, 18,26).

			Weeks (Mean)					p	p
	Group	**n**	**0**	**2**	**8**	**16**	**26**	**within**	
IPSS-1 incomplete emptying	Me180	10	2,60	2,80	2,30	2,50	2,20	0.76	
	tamsulosin	8	3,13	2,88	2,38	2,13	2,25	0.09	0.42
IPSS-2 frequency	Me180	10	2,40	2,30	2,10	2,10	1,70	0.33	
	tamsulosin	8	2,75	2,88	2,63	2,50	2,63	0.78	0.93
IPSS-3 intermittency	Me180	10	2,00	1,90	1,80	1,50	1,60	0.24	
	tamsulosin	8	2,63	2,00	2,38	2,00	2,25	0.39	0.53
IPSS-4 urgency	Me180	10	2,40	2,30	1,90	1,70	1,90	0.09	
	tamsulosin	8	2,38	2,13	2,00	1,63	1,75	0.29	0.79
IPSS-5 weak stream	Me180	10	3,90	3,50	2,90	3,10	3,10	0.013	
	tamsulosin	8	3,63	3,25	3,13	2,63	2,50	0.20	0.96
IPSS-6 straining	Me180	10	1,50	1,60	1,30	1,30	1,10	0.33	
	tamsulosin	8	2,38	1,63	1,50	1,38	1,63	0.23	0.69
IPSS-7 nocturia	Me180	10	2,10	1,70	1,60	1,40	1,50	0.007	
	tamsulosin	8	2,25	1,88	1,75	1,75	1,75	0.015	0.86
IPSS total	Me180	10	16,90	16,10	13,90	13,60	13,10	0.03	
	tamsulosin	8	19,13	16,63	15,75	14,00	14,75	0.10	0.48

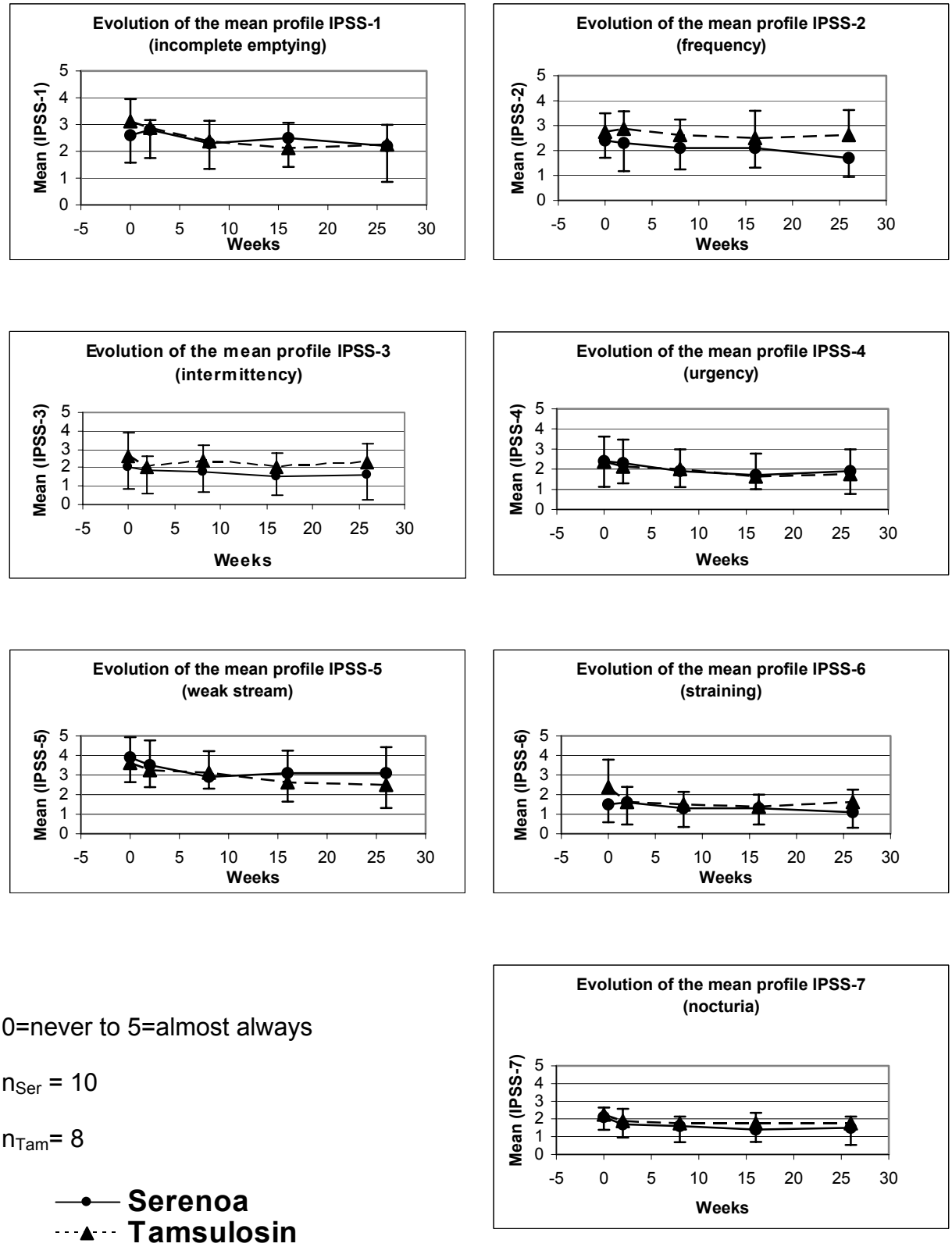

Figure IV- 7 Evolution of individual IPSS questions in both treatment groups with time.

Quality-of-life score

No significant differences were found between the quality-of-life score of the two treatment groups comparing means of values and means of paired differences “26 minus 0 weeks” with reached probability (p=0.62 based on Kruskal-Wallis test). The absolute value of the score slightly declined within the two treatment groups as shown in Figure IV-8 but did not reach statistical significance.

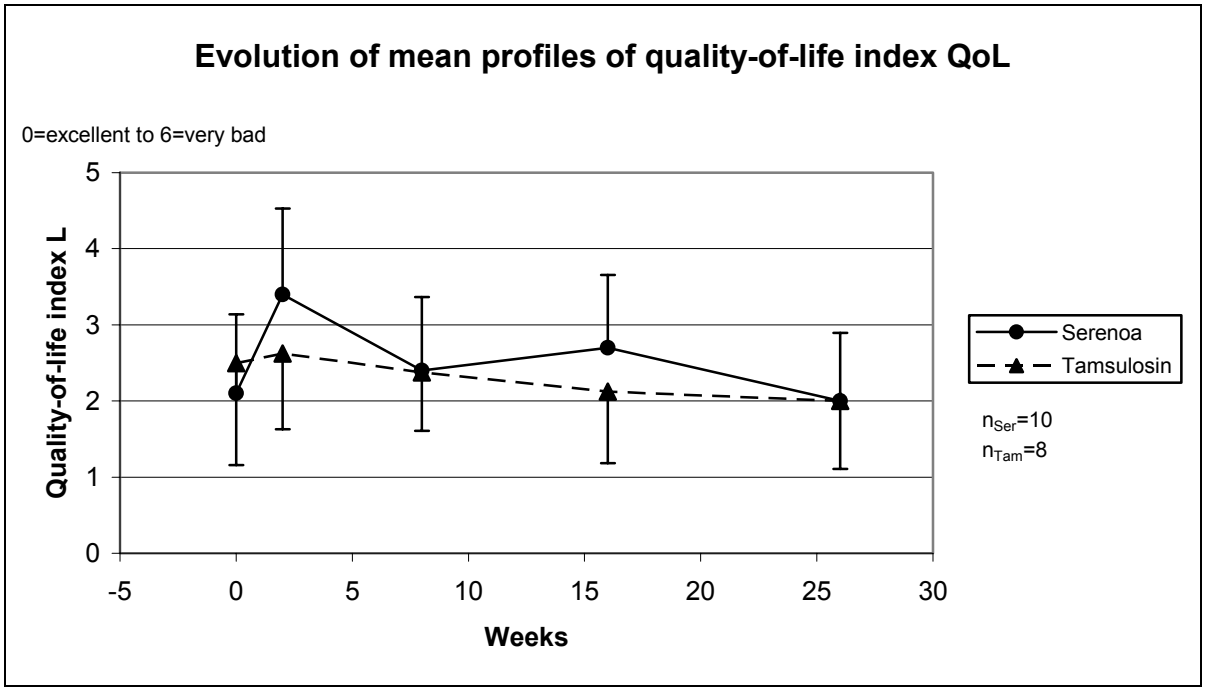

Figure IV- 8 Evolution of quality of life QoL means ± 95% CI for of treatment groups Me180 and tamsulosin at five control time-points (weeks 0, 2, 8, 16, 26).

4.4.2 Peak urinary flow rate (Qmax)

The peak urinary flow rates for the first two visits were averaged to give the baseline rate. All peak urinary flow rate measures were for a minimum urine volume of 150ml. Again, concerning the peak urinary flow rates, no significant differences were found between the two treatment groups comparing means of values and means of paired differences “26 minus 0 weeks” with a reached probability of p =0.42 based on Kruskal-Wallis test . The evolution of Q_{max} plotted over time is shown in Figure IV-9.

Within the two treatment groups up to week 8 a modest improvement with tendency to significance (p=0.056) was noticeable for Me180 and with slight significance (p=0.033) for the tamsulosin group. However, after week 8 this improvement was reversed in both treatment groups.

Comparing the absolute differences from baseline to endpoint the tamsulosin group showed a stronger increase with 3.99ml/s (51%) whereas the Me180 group showed only 0.89ml/s (10%). However, these differences where not significant.

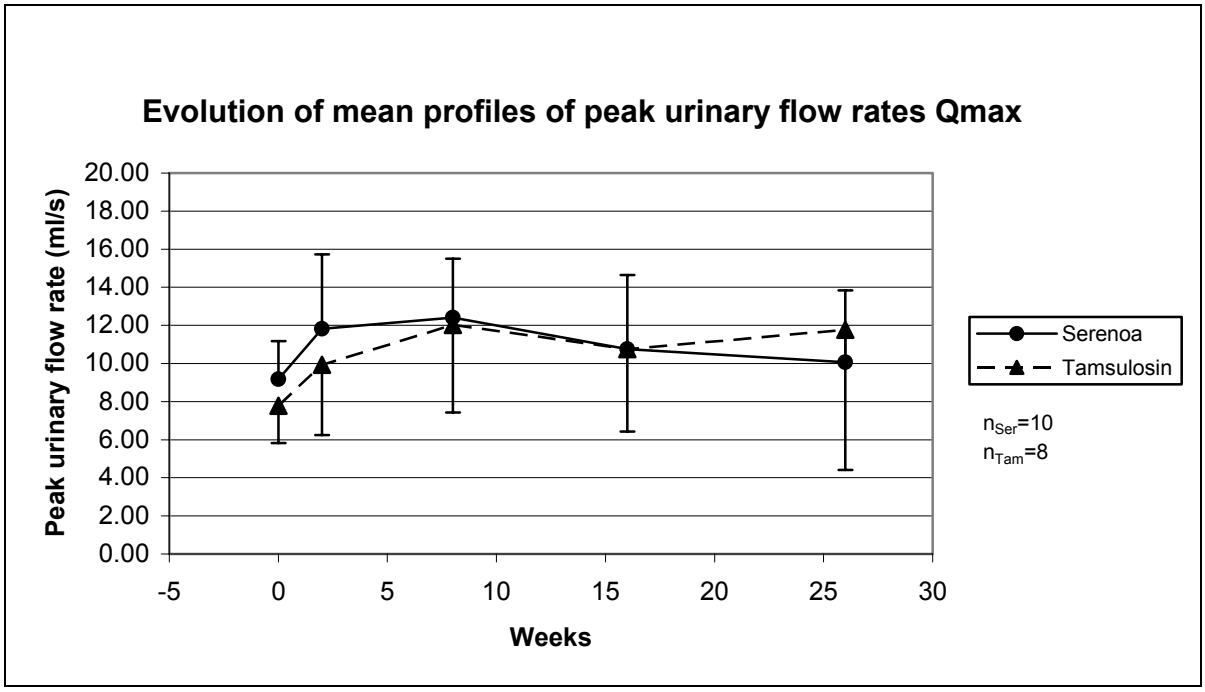

Figure IV- 9 Evolution of means ± 95% CI for peak urinary flow rates Qmax of treatment groups Me180 and Tamsulosin at the five control time-points (weeks 0, 2, 8, 16, 26)

In Figure IV-10 and IV-11 the evolution of mean Q_{max} values over time of each patient is depicted. Values of visits at week 2, 8 and 16 are missing in one patient from the Me180 group.

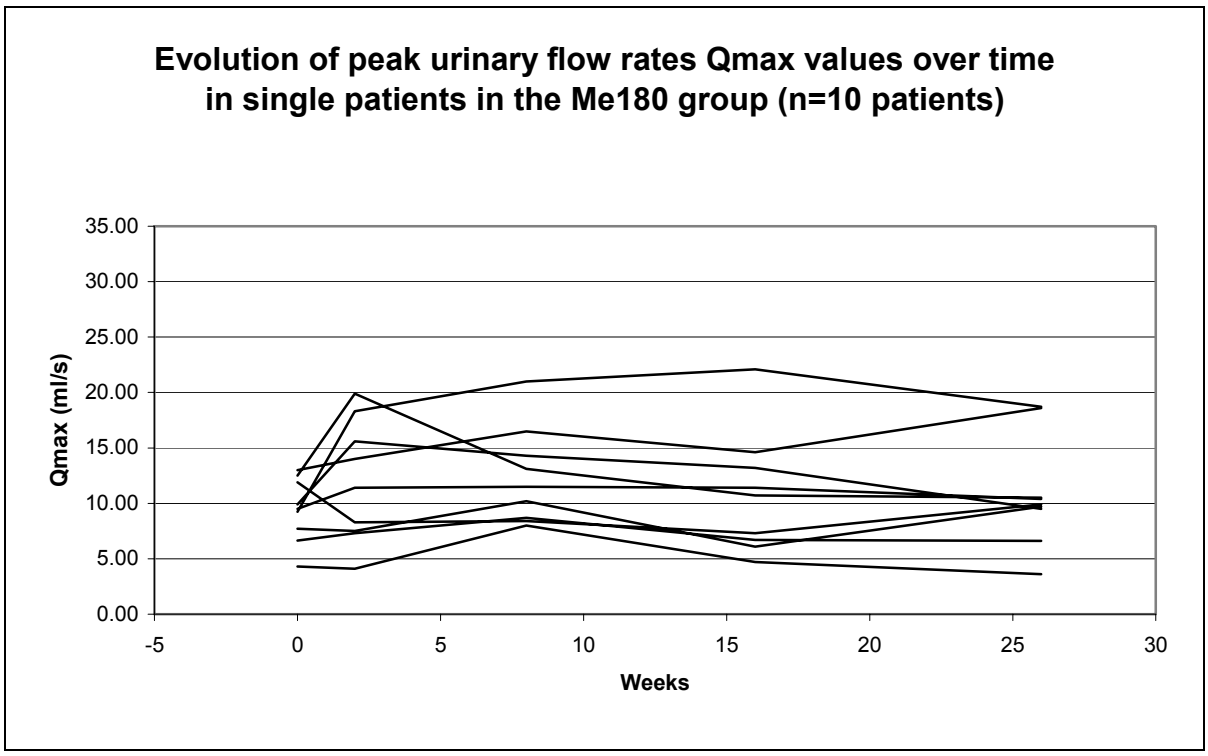

Figure IV- 10 Evolution of peak urinary flow rate (Qmax) values over time (26 weeks) in single patients in the group Me180 (n=10 patients)

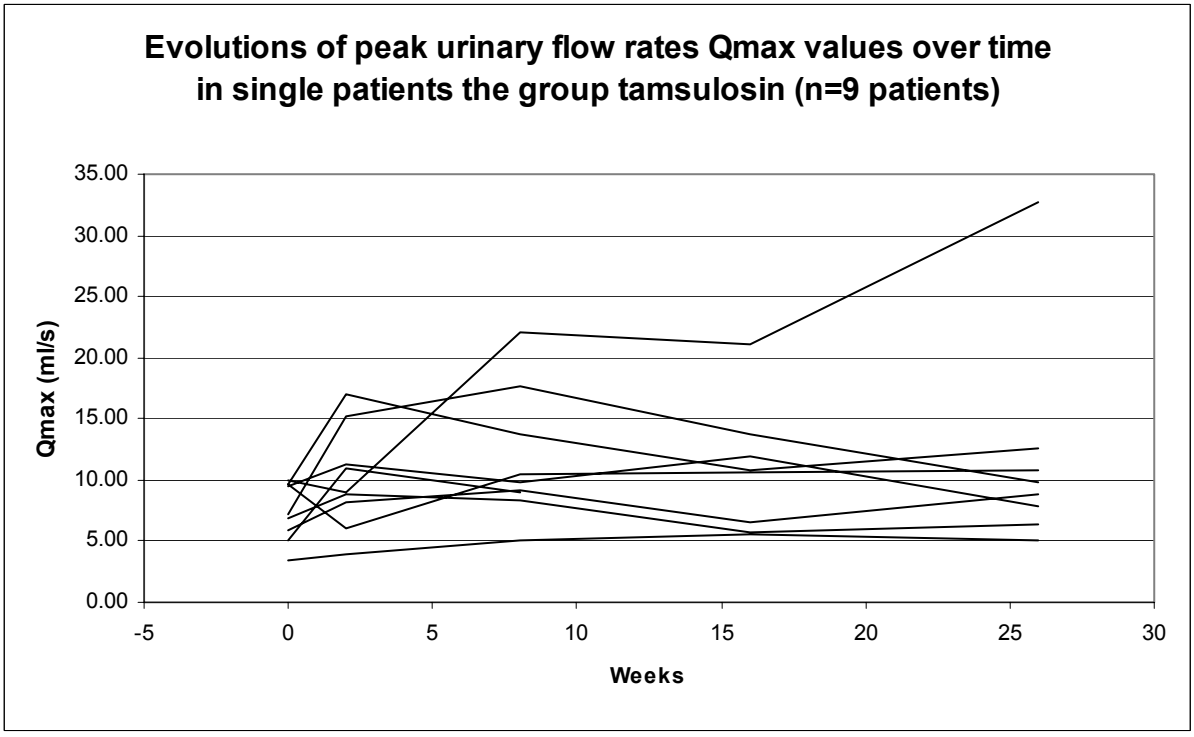

Figure IV- 11 Evolution of peak urinary flow rate (Qmax) values over time (26 weeks) in single patients in the group tamsulosin (n= 9 patients)

4.5 Secondary outcome measures of efficacy

4.5.1 Mean urinary flow rate

After six months of treatment, no significant differences were found between the two treatment groups comparing mean values of mean urinary flow rate Qave and means of paired differences “26 minus 0 weeks” with reached a probability p = 0.25 based on Kruskal-Wallis’ test (Figure IV- 12).

Within the two treatment groups no significant mean differences were observed (p=0.68 for Me180 and p=0.12 for tamsulosin), however evolution of both treatment groups were similar to that of Qmax (Figure IV-9).

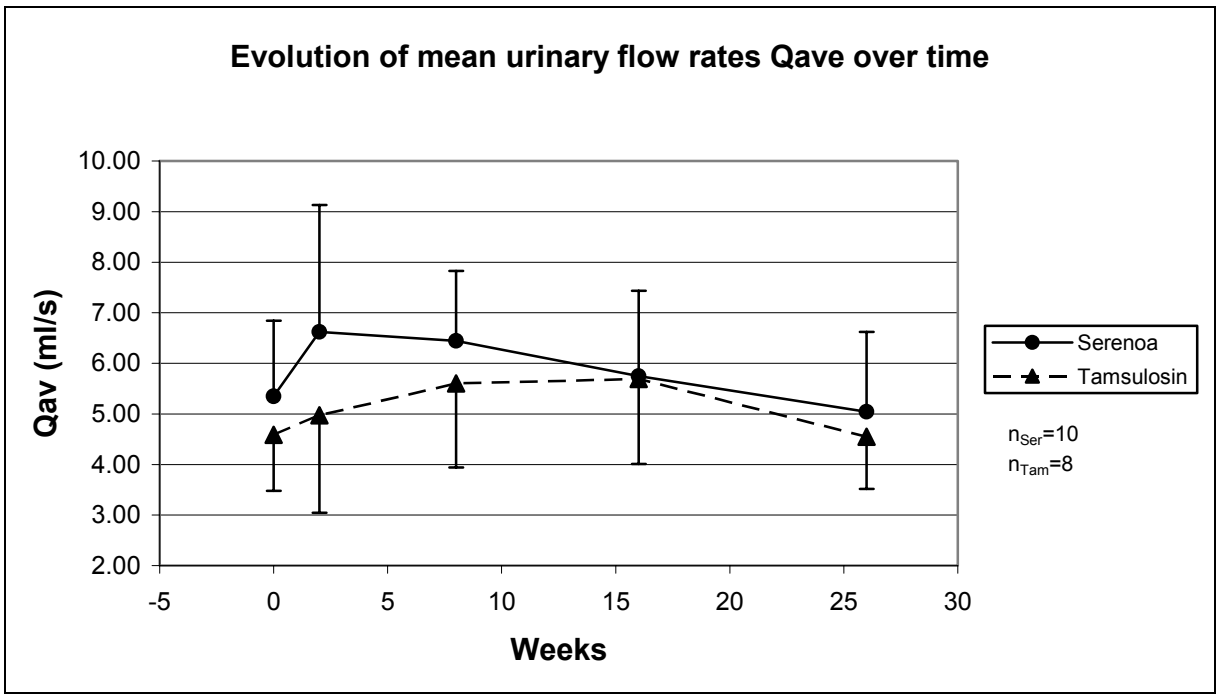

Figure IV- 12 Evolution of means ± 95% CI for mean urinary flow rates Qave of treatment groups Me180 and tamsulosin at the five control time-points (weeks 0, 2, 8, 16, 26)

4.5.2 Postvoid residual urine volume

No significant changes were found between the two treatment groups comparing means of values and means of paired differences “26 minus 0 weeks” (p>0.33 based on Kruskal-Wallis’ test)(Figure IV- 13).

In the tamsulosin group the mean was increasing up to week 8, but decreased again at week 16 and 26. Within the two treatment groups no significant mean modification was demonstrated (p=0.68 for the Me180 group, and p=0.10 due to the increase in the tamsulosin group, based on t-test).

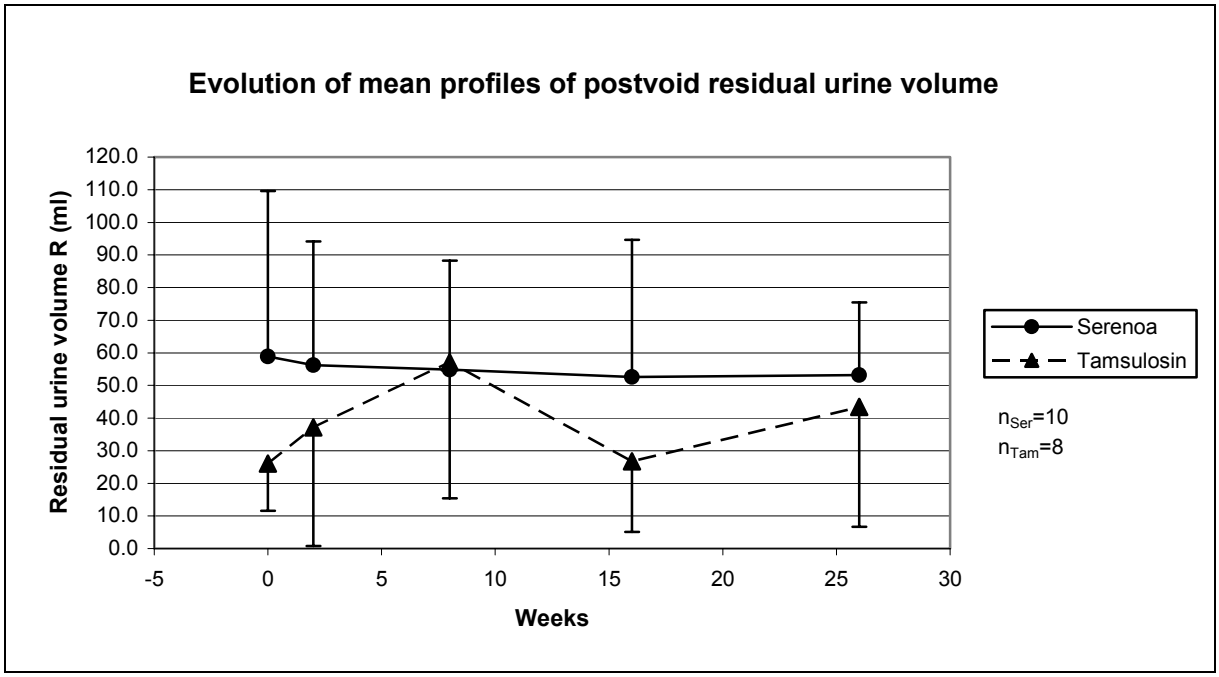

Figure IV- 13 Evolution of means ± 95% CI for postvoid residual urine volume of treatment groups Me180 and tamsulosin at the five control time-points (weeks 0, 2, 8, 16, 26).

In Figure IV-14 and IV-15 the evolution of postvoid residual urine volume values over time of the single patients are depicted. Values of visits at week 2, 8 and 16 are missing in one patient from the Me180 group.

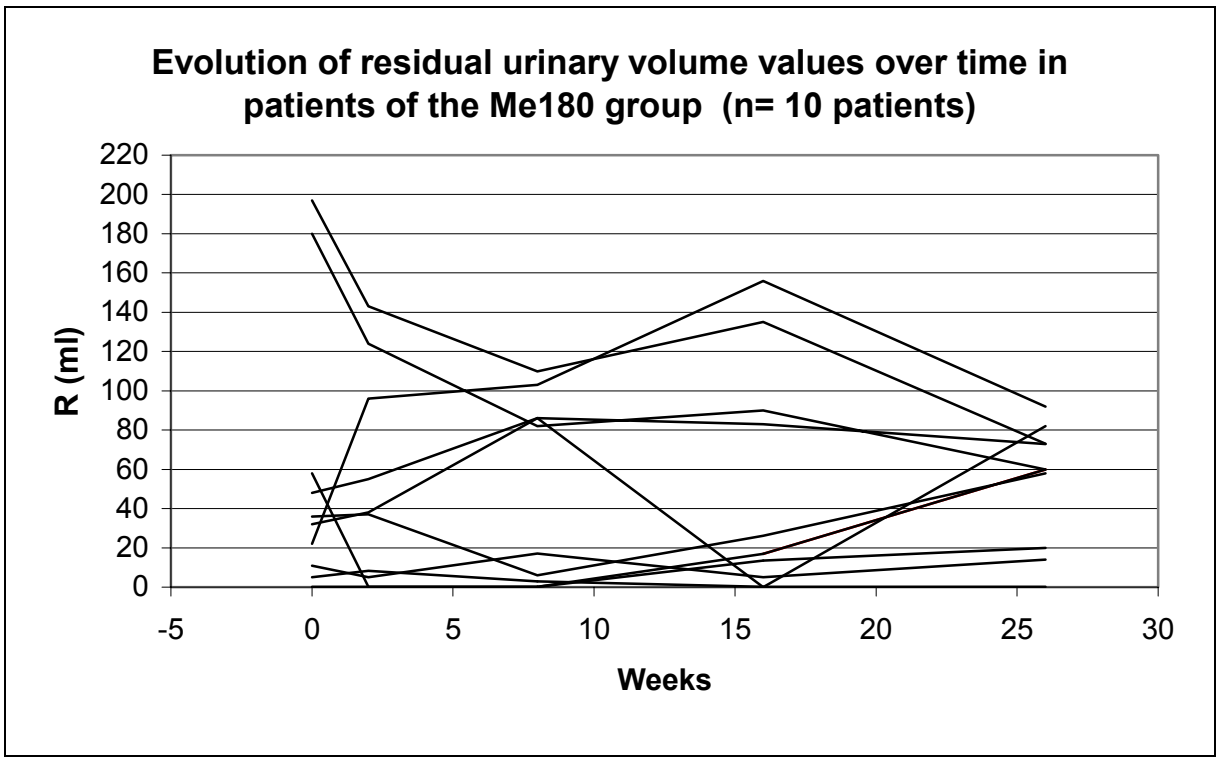

Figure IV- 14 Evolution of residual urinary volume values over time (26 weeks) in patients of the group Me180 (n=10 patients)

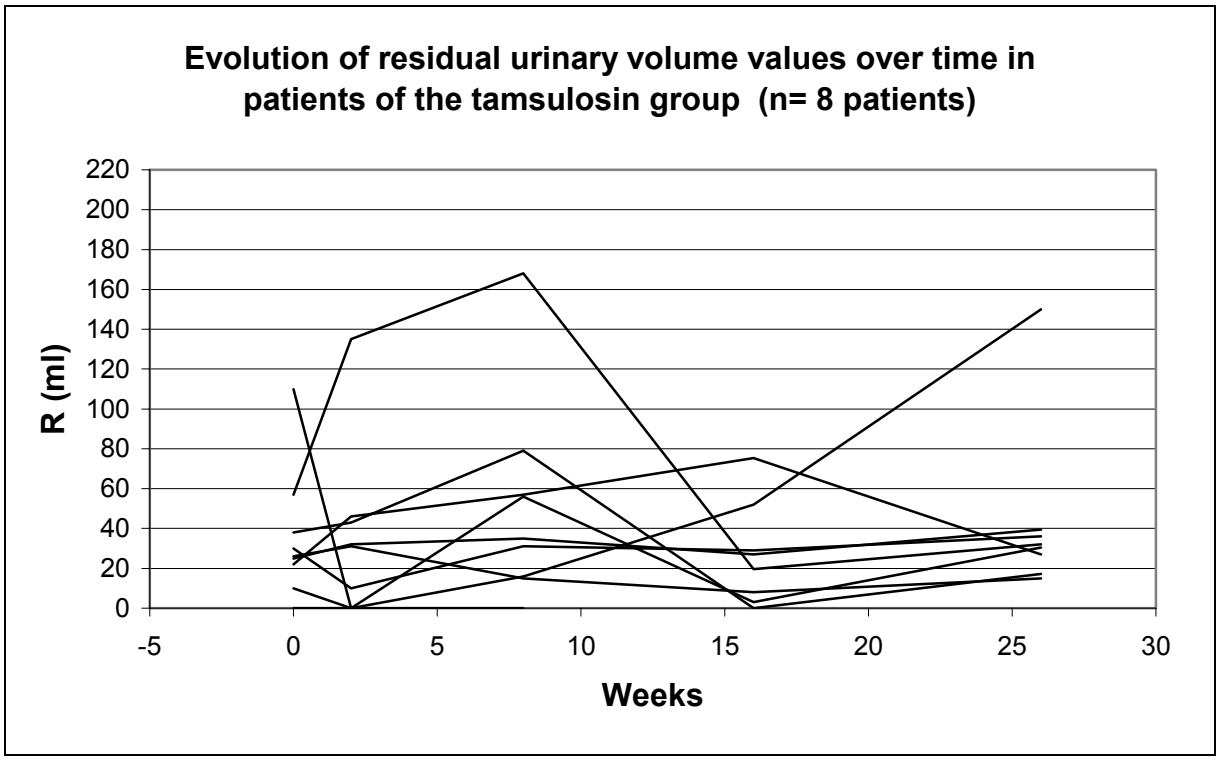

Figure IV- 15 Evolution of residual urinary volume values over time (26 weeks) in patients of the tamsulosin group (n=8 patients)

4.5.3 Prostate volume

The mean prostate volumes of both treatment groups at baseline and endpoint are shown in Table IV-6. No significant (p=0.59) differences were found between the two treatment groups comparing means of values and means of paired differences “26 minus 0 weeks”. Within the two treatment groups no significant mean modifications could be observed, with p=0.72 in the Me180 group and p=0.77 in the tamsulosin group.

4.5.4 International Index of Erectile Function (IIEF)

The mean evolution in the tamsulosin treatment group is significantly higher than in the Me180 treatment group (p=0.02). Comparing means of paired differences “26 minus 0 weeks” of the two treatment groups no significant differences were found (p>0.1). In none of the two treatment groups significant mean modifications could be observed: p= 0.10 within the Me180 group and p=0.19 within the tamsulosin group. However, absolute mean values decreased in both treatment groups.

4.6 Overall question

Additionally, the patient himself and the physicians were asked a global subjective question to determine the overall improvement (Table IV-8 and IV-9) and the tolerability (Table IV-10). There was no significant difference between the two treatment groups (p>0.1).

Table IV- 8: Overall improvement assessed by physician:

	Me180	Tamsulosin
very good	1 (10.0%)	1 (12.5%)
good	0 (0.0%)	0 (0.0%)
moderate	2 (20.0%)	1 (12.5%)
poor	3 (30.0%)	2 (25.0%)
none	4 (40.0%)	4 (50.0%)
total	10 (100%)	8 (100%)
p=0.96 (Fisher’s Exact Test)		

Table IV- 9: Overall improvement assessed by patient:

	Me180	Tamsulosin
very good	0 (00.0%)	1 (12.5%)
good	3 (30.0%)	1 (12.5%)
moderate	2 (20.0%)	3 (37.5%)
poor	3 (30.0%)	1 (12.5%)
none	2 (20.0%)	2 (25.0%)
total	10 (100%)	8 (100%)
p=0.73 (Fisher's Exact Test)		

Table IV- 10: Overall tolerability assessed by physician:

	Me180	Tamsulosin
very good	8 (80.0%)	7 (77.8%)
good	1 (10.0%)	1 (11.1%)
moderate	1 (10.0%)	0 (0.0%)
very poor	0 (00.0%)	1 (11.1%)
total	10 (100%)	9 (100%)
p=0.47 (Fisher's Exact Test)		

In the tamsulosin group one patient withdrew from the study because of strong dizziness. His medication tolerability was estimated to be very poor. The patient exhibiting moderate tolerability out of the Me180 group reported repeated dizziness. The patient himself estimated the medication tolerability to be moderate.

4.7 Safety results

4.7.1 Vital signs

Treatment affected neither systolic (SBP) nor diastolic blood pressure (DBP) nor pulse rate. No significant changes were found in intra- and inter group comparison of the two treatment groups in pulse and blood pressure measurement after sitting 5' and subsequently standing up ($p > 0.10$).(Table IV-11)

Table IV- 11: Means for pulse systolic (SPB) and diastolic blood pressure (DBP) over time

		Weeks (Mean ± SD)				
Parameter	**Group (n)**	**0**	**2**	**8**	**16**	**26**
Pulse (1/min)	Me180 (10)	76.8±10.2	74.9± 6.1	77.2±11.6	74.5±12.9	73.8±10.9
	tamsulosin (8)	71.9± 9.0	67.8± 6.0	71.8± 8.8	71.9± 9.0	70.8 ± 8.1
SBP after sitting 5' (mmHg)	Me180 (10)	135.3±13.9	138.1±14.2	138.0±13.5	134.6±12.9	130.9±12.4
	tamsulosin (8)	136.4± 17.2	126.6± 15.3	134.0± 20.9	133.5± 10.3	128.4 ± 1.7
DBP after sitting 5' (mmHg)	Me180 (10)	83.4± 10.2	83.4± 11.1	82.5± 6.6	87.7± 14.0	82.7 ± 7.9
	tamsulosin (8)	82.0± 11.4	82.5± 8.7	80.8± 9.9	81.0± 4.6	78.1 ± 5.1
SBP after standing (mmHg)	Me180 (10)	135.1± 13.2	134.4± 12.7	137.5± 13.9	139.4± 13.4	134.2± 10.6
	tamsulosin (8)	135.9± 19.0	125.0± 12.4	140.0± 21.2	140.9 ± 6.9	130.6± 15.6
DBP after standing (mm Hg)	Me180 (10)	84.6± 9.9	82.2± 6.7	87.4± 9.6	88.8 ± 9.1	85.6 ± 7.1
	tamsulosin (8)	87.9± 12.8	82.4± 10.1	85.5± 11.3	87.9 ± 10.0	82.8 ± 5.2

4.7.2 Laboratory parameters

A panel of 6 standard blood tests (alkaline phosphatase, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), haemoglobin, creatinine, and prostate specific antigen (PSA)) was assessed at baseline and endpoint (Table IV-12). Within the two individual treatment groups no significant changes were found between baseline and endpoint (p> 0.01).

The baseline mean of alc. phosphatase in the Me180 group was significantly higher compared to the tamsulosin group (p=0.026). This might explain why, at the endpoint, the mean of the Me180 group decreased compared to baseline and within the tamsulosin group the mean increased from baseline to endpoint with a tendency to significance (p=0.07). However, between the two treatment groups no significant changes were found for the 6 laboratory parameters (p> 0.01).

Table IV- 12 Means for laboratory parameters of treatment groups Me180 and Tamsulosin at controls 0 and 26 weeks as means of paired differences "26 minus 0 weeks" with reached probability p based on the Kruskal-Wallis test

			weeks (means)		
Parameter	**Group**	**n**	**O**	**26**	**p**
Alc. phosphatase	Me180	10	87,5	84,5	
(U/l)	tamsulosin	8	60,9	66,1	0,07
ASAT	Me180	10	27,6	27,2	
(U/l)	tamsulosin	8	24,8	25,9	0,35
ALAT	Me180	10	29,2	30,6	
(U/l)	tamsulosin	8	27,5	29,3	0,86
Creatinine	Me180	10	79,2	79,2	
(umol/l)	tamsulosin	8	78,0	81,3	0,37
Hemoglobine	Me180	10	15,44	15,18	
(mg/dl)	tamsulosin	8	14,86	14,90	0,31
PSA	Me180	10	1,83	1,39	
(ng/ml)	tamsulosin	8	1,16	0,99	0,65

4.7.3 Comorbidity and comedication

At baseline 4 patients in the Me180 group and 2 patients in the tamsulosin group reported comorbidity and comedication accepted during the study treatment. (Table IV-13).

Table IV- 13 Comorbidity and comedication at baseline

	comorbidity	comedication
Me180 group	enhanced intra-ocular pressure	Latanoprost (Xalatan®) once daily
	eczema and neurodermatitis	cutaneous fluticasoni propionas (Cutivate crème) and Clobetasoli-proprionas (Dermovate Crème)
		Pitosan shell extract
	previous arthritic pain	Ginkgo extract (Ginkgo vital)
	hepatitis C carrier since 30 years	
tamsulosin group	enhanced cholesterol level	Atorvastatinum 10mg(Sortis®) once daily
	previous polymyalgia	Magnesii omeprazolum 20mg (Antra®) once daily

In Table IV-14 comorbidity and comedication of patients are shown, of which a relationship with the active drug was excluded.

Table IV- 14: Comorbidity and comedication during study

	comorbidity	comedication
Me180 group	headache	paracetamol 250mg , propyphenazoum 50mg (Saridon®) each 2nd week against headache
	neurodermatitis, known	triamcinolon 4mg (Kenacort®) reductive therapy scheme over one week
	cold	Triofan® nose spray(xylometazolinum, carbocisteinum) for 3 days
	headache	mefenacidum 500mg (Ponstan®) two times
	allergic rhinitis, known	mometasoni 50ug/d (Nasonex®)
	calf cramp, known	magnesium 300mg (Magnesium Diasporal ®)
Tamsulosin group	cold	acidum salicylicum 500mg (Alca C®) 3 times taken
	sore throat	amoxicilin 1000mg (Aciclav®) for 6 days
	headache	mefenacidum 500mg (Ponstan®)
	diarrhoea once in vacation	charcoal tablet 1x
	sleeping trouble once in vacation	zolpidem 10mg (Stilnox®) 1x

4.7.4 Adverse events and withdrawal from treatment

Adverse effects, of which a relationship with the treatment could not be excluded, were reported in 3 out of the 10 patients in the Me180 group and in 4 out of the 8 patients in the tamsulosin group (Table IV-15). Severity was mild to moderate for all events. There was no serious adverse effect reported. Only one patient in the tamsulosin group withdrew from the trial because of continuous strong dizziness, this patient already reported stomach problems in the first period (study begin to control 1), in the second period (control 1- 2) he started to feel dizzy and stopped study participation in the third period (control 2-3). All other events resolved without treatment discontinuation.

The most frequent adverse events in the tamsulosin group were ejaculation disorders (n=3). Other adverse events not resulting in withdrawal included occasional dizziness, and occasional headaches in the same patient, and reduced ejaculate volume, or absence of ejaculate volume.

Three patients in the Me180 group reported mild adverse effects with a possible association (Table IV-15). These were non-serious and additional medication or interruption of therapy was not required. Beyond the drop-out patient in the tamsulosin group there were three patients reporting mild to moderate adverse effects (Table IV-15):

Table IV- 15 adverse effects of which a relation to active drug can not be excluded

Treatment group	Patient number	Mentioned adverse effects by patients
Me180 group	No.9	– throw ups after intake without breakfast, temporary
	No.15	– dizziness, a few times temporary, disappeared after contr. 3
	No.17	– flatulence, temporary
tamsulosin group	No.4	– decrease ejaculatory volume, mild but continuous, likely associated with study treatment
	No.5	– pyrosis at control 1, dizziness in the morning at control 2, discontinuation of the study because of continuous strong dizziness
	No.12	– repeatedly reported decrease ejaculatory volume, swollen eyes, reduced libido, which may have been associated with study treatment
	No.13	– reduced ejaculatory volume, during one period, possibly associated with the study treatment

Patients receiving tamsulosin experienced a statistically significant (p=0.013) higher occurrence of adverse events listed compared to Me180 group as shown in Table IV-15.

5 Discussion

In this second, clinical part, a controlled randomised double-blind pilot study was conducted to compare the therapeutical effects and safety of the over-the-counter *Serenoa repens* extract Me180 with tamsulosin, a selective $\alpha 1_A$-blocker commonly used in patients with symptomatic obstructive BPH. As mentioned before, although this extract is already commercially available, it has not been investigated in patients with BPH so far. The outcome of the present pilot study is intended to form the basis for the design of an extended study with more patients, to assess the efficacy of the extract over a longer treatment period.

This study was exploratory and with the included 19 patients not sufficiently powered to specifically test the efficacy of *Serenoa repens* extract Me180 compared to tamsulosin. Therefore, our results may only indicate a tendency for a non significant difference between the treatments over the 26 weeks. During the treatment period most of the participants had some improvements in their symptoms of BPH but there was no significant beneficial effect of both treatment groups over the 26-week treatment time. These effects were not as pronounced as reported in previous studies, particularly for the IPSS and peak urinary flow, which apparently improved with *Serenoa repens* extract (SRE) treatment and tamsulosin treatment, as assessed in two meta-analyses by the Cochrane Library (Wilt et al 2002a; Wilt et al 2003). Moreover, Debruyne and co-workers demonstrated that a *Serenoa repens* hexane extract (Permixon®) and tamsulosin are equivalent in the medical treatment of lower urinary tract symptoms in 542 men with BPH, during and up to 12 months of therapy (Debruyne et al 2002).

Obviously, these findings provoke the question of why the investigated preparation failed to confirm results from this previous trial. Study medication capsules were returned and counted at every control and we consider that the compliance with over 90% was good. Explanations for the lack of improvement could be that there were too few patients, or that the duration of 26 weeks was insufficient to detect any significant change.

5.1 Number of patients and recruitment

As shown in Figure IV-1 we intended to include 70 patients in the pilot study. The patient recruitment was stopped because of a high dropout rate at the telephone interview (74%) but also at the first visit (82%), so that only 4.5% of interested participants were finally included and randomised in the treatment groups (Table IV-3 and Table IV-4).

The study was conducted according to the guidelines of 4th International Consultation on BPH (1997) (Roehrborn 1998), which established guidelines to standardise the trials and to allow a comparison of them. Strict inclusion and exclusion criteria were applied to form a homogeneous patient group, to ensure that the study population represented obstructive BPH, and to avoid interferences with other drugs.

On one hand, without a solid definition of the diagnosis of BPH, it can be difficult to interpret the results of clinical trials for effectiveness; on the other hand applying to strict inclusion criteria, may lead to a high exclusion rate of patients, making it difficult to recruit enough patients for the study. Unexpectedly many men responded to our advertisements (420 patients to 6 advertisements). This might be because many men have not sought medical advice, accepting LUTS as part of ageing, but with increased publicity about men's health and raised awareness of prostate cancer, more men are seeking help. Nevertheless, 205 patients (48.8%) were not invited after the telephone interview because of concomitant medication not accepted during the study treatment. A large proportion of men with LUTS have already cardiovascular, diabetic and/or prostatic medication being exclusion criteria in the present pilot study. This may possibly be due to the extended health care system in Switzerland. Another explanation for the problems in patient recruitment is that participants were recruited in the region of Basel, in a region with a high density of medical doctors (357 medical doctors per 100 000 inhabitants in Basel – City compared to 137 per 100 000 inhabitants practising in Central Switzerland (Bundesamt für Statistik 2001)). It remains an open question, if more patients could be invited for the first visit in other regions with less medical doctors. However, one has to consider that the selected co-medication inclusion and exclusion criteria are according to the recommendations of the Consultation on BPH (1997), but also to the supplier recommendation mainly of importance in the case of tamsulosin.

5.2 Impact of the treatment on symptoms and quality-of-life

The establishment of symptoms is only one facet of the response to treatment of obstructive BPH, although an extremely important one from the patient's perspective.

During the treatment time, most of the participants had some improvement in their symptoms of BPH but there was no significant beneficial effect of both treatments during 26 weeks. The lack of a significant improvement within the treatment groups, particularly for the tamsulosin group was somewhat surprising. Up to week 16, a slight significant improvement was shown in the Me180 group and an improvement in the tamsulosin group with a tendency to significance. However, compared to literature, the decline from baseline in the absolute mean value of the total IPSS is poor.

Most of the tamsulosin studies used a different symptom score making a percentage comparison difficult. However, many studies reported an improvement of the IPSS even after a short time of treatment with tamsulosin (Michel et al 2001; Wilt et al 2003). For the *Serenoa repens* hexane extract, Permixon®, a IPSS reduction of 37% after 26 weeks was reported (Carraro et al 1996). In a further trial with Permixon® and tamsulosin, a somewhat smaller IPSS reduction was seen with 26.7% and 27.5 respectively, after 12 months. However maximum improvement of symptom score was already achieved after 3 months in both treatment groups (Debruyne et al 2002). These improvements in symptom score are greater with respect to reduction than our observed 22.5% in the Me180 group. We suggest that the amount of 10 and 8 patients, respectively, within in the two groups was not sufficient to show any significance.

The initial tendency of improvement could be explained by the start motivation for the therapy of the patients. It has been shown in literature that during the placebo run-in-period, the mean total IPSS decreased from a mean of 16.8 to 15.3 (Debruyne et al 2002). There is considerable evidence to support the belief that a significant placebo effect is manifest in trials for treatments of BPH (Nickel 1998). It appears that simply having some medical attention and talking about their condition was therapeutic for these men. In further studies, this run-in-phase improvement has to be taken in account, by inclusion of a placebo-run-in- phase.

In other recent trials by Willets and co workers with a CO_2 *Serenoa repens* extract, no significant improvement over 12 weeks and in comparison to placebo treatment was reported (Willetts et al 2003). The lack of improvement was explained by a relative low mean baseline IPSS 14.1 for SRE and 17.9 for placebo. It has been previously established, that the magnitude of IPSS changes during medical therapy, is related to the baseline score: the higher the initial score, the greater the decrease (Denis et al 1998). In the present study, however, the mean baseline of 16.9 in the Me180 group and 19.1 in the tamsulosin group was comparable to earlier reports of trials investigating SRE's: 15.3 (Debruyne et al 2002) , 15.7 (Carraro et al 1996), 17.4 (Marks et al 2000) and 17.7 for tamsulosin (Michel et al 2001).

LUTS can be divided into obstructive and irritative symptoms. The former occur during the bladder emptying phase and include hesitancy, decreased stream, feeling of incomplete emptying, straining to void, intermittency, postvoid dribbling, and urinary retention. The latter occur during the storing phase and include daytime frequency, nocturia, urgency, and urge incontinence.

Non significant differences were observed between the two treatment groups at 26 weeks in terms of irritative symptoms (p=0.93) and obstructive symptoms (p=0.53) (Figure IV-5 and IV-6). However, within the treatment groups, the Me180 group showed a slight significance of decrease in irritative symptoms (p=0.03) and a tendency to significance for a decrease of obstructive symptoms in both treatment groups (0.07).

Although obstructive (voiding) symptoms are most prevalent, irritative (storage) symptoms are considered to be the most bothersome for the patient, interfering to great extent with activities of daily life, and having therefore a considerable negative impact on quality-of-life (Djavan 2003). They may cause social embarrassment or social isolation because the patient may no longer undertake long journeys or activities, where poor access to lavatories may be a problem. The improvement particularly of irritative symptoms may therefore have a beneficial effect on quality-of-life for these patients. However, no significant changes were assessed during the 26 week treatment time in any of the two treatments. The initial worsening at week 2 in the Me180 group cannot be explained. In the tamsulosin group QoL remained on the same level over the treatment time.

Mortality from benign prostatic enlargement is very rare and other sequelae such as acute urinary retention, severe urinary tract infection and acute renal failure are increasingly rare (MacDonald & McNicholas 2003). The main focus for the vast majority of men is the effect of the drug treatment on symptoms and on their quality-of-life. Since the impact of LUTS on the patient's quality-of-life is highly variable and not directly related to any measurable physiological factors, the patient's perception of the severity of the condition, as well as the degree to which it interferes with his lifestyle or causes embarrassment, should be the primary consideration in choosing therapy.

5.3 Urinary flow rate, residual urine and prostate volume

While the improvement of the symptoms are clearly most important to patients, indirect benefits such as changes in urinary flow rate, postvoid residual urine volume, and prostate size are useful for comparing different treatment modalities.

5.3.1 Urinary flow rate

Concerning peak urinary flow rates, no significant differences were found between the two treatment groups (Figure IV-9). Within the two treatment groups up to week 8 a modest improvement with tendency to significance was noticeable, however, after week 8 this improvement was reversed in both groups.

This initial improvement could be explained by a learning effect. It is to consider that patients may improve their performance from measurement to measurement. Moreover, controversy exists regarding the reproducibility of this parameter in the same individual on repeated measurements. Golomb and co workers studied the variability and circadian changes in 32 men with BPH by repeated home flow rate recording (total number of recordings 476, average per subject 15) (Golomb et al 1992). In 87.5% of patients the peak flow rate varied by at least one standard deviation, and in 47% by two. To prevent the influence of diurnal and other variations, all visits were performed in the morning and the first two Q_{max} were averaged for the definition of obstructive BPH (Abrams 1998). However, for the execution of the study, it was not possible to perform all flow studies in by double measurements.

There are some more aspects of flow rate recording which are of immediate relevance for the outcome reporting of BPH treatment: The peak flow rate was based

on a minimum voided volume of 150ml to ensure that no patient was included in whom the low peak flow was the result of an inadequate voided volume. However, during the trial this minimum volume of 150ml turned out to be a problem for many patients leading them to excessive drinking and subsequently to potentially artificial values.

A meta-analysis of tamsulosin showed that the mean change from baseline in peak urine flow of 1.1 ml/sec (95% CI = 0.59, 1.51) for 0.4 mg (Wilt et al 2003). In a larger comparative trial of Debruyne et al (2002) the increase in Q_{max} was similar in both treatment groups with 1.8ml/s for Permixon® and 1.9ml/s tamsulosin. Interestingly these small increases led to significant improvement of LUTS.

5.3.2 Residual urine volume

Concerning postvoid residual urine volume, no significant changes were found within and between the treatment groups during the study. However, the evolution in Figure IV-13 shows that the tamsulosin group showed much lower residuals at baseline than the Me180. As mentioned earlier, because of the lack of enough eligible patients, the design was adjusted from 30ml<R<200ml to R< 200ml. This might have led to a loss of homogeneity in the patient collective.

The evolution of mean values shows a strong variability within the tamsulosin group (Figure IV-13). However, values of single patients (Figure IV-14 und IV-15) show strong variability in both treatment groups. This variability has been investigated earlier by Griffiths and co workers showing large within-patient variability because of large systematic variations with time of the day, with greatest volumes in the early morning (Griffiths et al 1996). To take circadian changes in account, in the present study all visits were performed in the morning.

5.3.3 Prostate Size

It is well established that the prostate size correlates only moderately with patients symptoms, perception of bother, or other commonly used parameters. There is only a poor correlation between the total symptom score and prostate volume (Barry et al 1993; Bosch et al 1995), and it was concluded by Barry et al. that the prostatic enlargement is not the critical factor in the production of either symptoms or physiological outlet obstruction. In some patients a relatively small degree of

strategically located hyperplasia in the periurethral area might cause considerable physiological obstruction, while in other individuals considerable hyperplasia can occur without producing obstruction (Barry et al 1993). Therefore, the measurement of the prostate size is only of secondary importance.

The mean prostate volume of both treatment groups at baseline and endpoint showed no statistically significant differences whether between the groups or within the groups. This is in accordance with previous results of other tamsulosin- and SRE-studies where only weak modulations of prostate volumes were found (Debruyne et al 2002; Wilt et al 2002a; Wilt et al 2003).

5.4 Treatment effects on sexuality

There was a significant difference in IIEF at baseline between the two treatment groups, however no change in the IIEF was observed within the groups during the 26-week treatment time. This is consistent with previous studies where the effect on libido and sexuality was compared in SRE and placebo treatment groups (Reece Smith et al 1986; Willetts et al 2003). To our knowledge, the IIEF questionnaire was so far not investigated in tamsulosin studies, however, there is agreement within the studies that tamsulosin exhibits an ejaculatory dysfunction (retrograde ejaculation) without affecting libido.

The IIEF as a questionnaire turned out not to be an ideal tool to test the effect on libido induced by the treatment. Most of the patients were not very interested in filling in these answers, particularly when they were not sexually active. Patients are seeking help because of their symptoms associated with BPH. The IIEF was found to be too extensive for the identification of treatment induced effects on sexuality. Generally, and mainly towards the end of the trial, it was noted that the patients felt less inclined to spend time completing the questionnaires accurately. The value of this questionnaire is limited by the sole focus on current sexual functioning, the superficial assessment of non-erectile components of sexual response like ejaculation and the limited assessment of the partner's relationship. Nevertheless, treatment effects on sexuality remains an interesting fact. In a larger trial, it should be possible to measure only those patients who are sexually active.

In the present pilot study 3 out of 8 patients in the tamsulosin group reported a reduced ejaculatory volume, whereas no patient of the Me180 group reported such

an effect. Ejaculation disorders have been reported to occur in patients treated with tamsulosin (Lepor 1998; Schulman 2001; Debruyne et al 2002). It is probably related to the mode of action of α_1-adrenoceptor antagonists (Schulman 2003).

5.5 Tolerability, adverse effects and safety

In general the treatment was well tolerated. Moderate tolerability was reported for only one patient in the Me180 group because of occasional dizziness, and in the tamsulosin group one patient withdrew from the study because of continuous strong dizziness in the morning. For this patient the tolerability was reported to be very poor.

The overall incidence of adverse events was higher in the tamsulosin group, with a slight significance compared to the Me180 group. Reduced ejaculatory volume was the most frequently reported event. As mentioned above, this is a known adverse effect of tamsulosin. This effect is usually not affecting patients' quality-of-life and is therefore well accepted by the patients when having a positive therapeutic effect. Concerning Me180-induced adverse effects, one patient reported throw ups after medication intake and another patient reported flatulence. However, both adverse effects occurred only at initiation of therapy. Other Me180 extracts were reported to cause mild gastrointestinal disturbances (Wilt et al 2002a).

The safety data published previously for other SRE's and for tamsulosin were confirmed (Wilt et al 2002a; Wilt et al 2003). In our pilot study, treatment with either Me180 or tamsulosin had no effect on serum PSA levels, consistent with earlier results with other SRE preparations and tamsulosin. This is in contrast to 5α-reductase inhibitors, such as finasteride and dutasteride (Andriole & Kirby 2003), which in addition to their 5α-reductase enzyme-inhibitory activities, alter the level of PSA expression by inhibiting the complex formed between androgen receptors and the steroid receptor binding consensus, a functional androgen-responsive element in the promoter region of the PSA gene (Wang et al 1997).

5.6 Data recording

During the pilot study, a new electronic case report form (eCRF), developed by pnn, pharma nation network AG, Zurich, was tested. Data were therefore not only recorded on conventional CRF (hard copy paper version) but also on electronic CRF. The layout of the eCRF was arranged according to the finished paper version. Otherwise, a more user-friendly design could have been chosen, appropriate to requirements of electronic acquisition such as less scrolling between templates that have to be filled in. Nevertheless, the eCRF revealed to be a convenient method for data recording. The advantages of the eCRF would be particularly notable in large multicenter trials: Data would be entered directly after the visits, and therefore, the conventional, doubled data entry after study termination could be omitted. Data analysis after study termination is thereby considerably simplified. For example, at each time point during the study in-between-evaluations are possible. Data entries from various study centres can be reviewed from anywhere. In addition, the actual status of the trial may be accessed and be clearly determined at any time point via a “status function”.

6 Limitations of the study protocol

The present study aimed to assess a significant improvement of the symptoms after 26 weeks, and to investigate the safety and tolerability of *Serenoa repens* fruit-extract Me180 (320mg/d) versus tamsulosin (0.4mg/d) as an active control for patients with obstructive benign prostatic hyperplasia. The efficacy of this *Serenoa repens* extract Me180 can only be assessed by a larger and extended study, powered to assess at least equivalence versus a reference-control or even superiority over placebo.

This present study was conducted as a two-arm, parallel-group, and active-controlled pilot study. No placebo arm was included, since it was not stated mandatory in the ICH guidelines. Nevertheless, the lack of a placebo arm, ultimately limits the possible judgement of efficacy of the drugs under study and the trial cannot be put into context with any other trials. Since only the equivalence of *Serenoa repens* extract Me180 versus reference should be assessed, it is therefore recommendable to additionally include a third placebo-arm.

We decided to conduct the first pilot study with a duration of 6 months. To document the efficacy, the 4th International Consensus on BPH recommended a duration of at least 12 months for clinical trials. Summarized data from several studies indicate that spontaneous improvement of symptoms may occur within 3-6 months, while in most cases deterioration takes a longer period of time (Schulze et al 1992). Therefore, it would be appropriate to prolong the treatment time to 12 months.

In the present study, we found an initial decrease of the symptom score with a tendency to significance. This confirms, the study of Debruyne and coworkers the 4 week placebo run-in-phase showed a significant decrease in the IPSS. Therefore, such a run-in-phase needs to be conducted with all treatment parameters (Debruyne et al 2002).

We had to stop our trial prematurely because of difficulties in patient recruitment. Most of the patients did not fulfil all inclusion criteria at the telephone interview because of concomitant medication which possibly could have could interfere with the study medication. However, one has to consider that extracts of *Serenoa repens* are usually given to a patient population which is taking these drugs anyway. A combination of phytotherapeutics with other medication may have possible

interactions, and this fact should be considered before treatment. However, this can only be judged when it is evaluated in clinical studies. It would therefore be a possible inclusion in order to reach this group of patients.

The IIEF revealed to be a too extensive questionnaire to assess associated changes in sexual function by the study medication, especially for patients not being sexually active. In a larger trial it should be possible to measure only those patients who are sexually active.

7 Comparison of the pilot study with previous Serenoa trials

Earlier reports have suggested a beneficial effect of SRE preparations mainly of one *Serenoa* hexane extract (Permixon®) (Champault et al 1984; Descotes et al 1995; Carraro et al 1996; Braeckman 1997; Debruyne et al 2002). The lack of a significant improvement of symptoms score and Q_{max} is consistent with earlier placebo-controlled studies by Reece Smith and co workers and Willets and co workers (Reece Smith et al 1986, Willetts et al 2003). In one study with 45 patients with LUTS suggestive of BPH being treated for 12 weeks, the α1-adrenoceptor inhibitor prazosin was significantly more efficacious than a SRE (Adriazola Semino 1992). Another study with 63 patients being treated for 3 weeks found that the α1-adrenoceptor antagonist alfuzosin reduced irritative and obstructive symptoms in the Boyarski score to a greater extent than SRE (Grasso et al 1995). Moreover, in an open-label study of 50 patients treated with SRE for 6 months, despite an improvement of symptoms, no significant improvement was noted in any urodynamic parameter: peak urinary flow rate, postvoid residual urine volume, detrusor pressure at peak flow, or Abrams-Griffiths number (Gerber et al 1998). However, all these trials were also of short duration and with a small number of patients, limiting the conclusion of these trials.

Combination therapy, SRE plus tamsulosin, did not provide an additional clinical benefit expected in individual patients submitted to the therapy (Glemain et al 2002). Neither did combinations of 5α-reductase inhibitor plus α-blockers provide any additional benefits (Lepor et al 1996; Debruyne et al 1998). This may be due to the magnitude of clinical benefit expected in individual patients.

The tolerability and safety of tamsulosin and various SRE's were reported to be good in all studies investigating one of these products. The only adverse event that occurred with a higher incidence with tamsulosin than with SRE was abnormal ejaculation (Schulman 2003), which is in accordance to the present pilot study.

8 Conclusion

Taking into account the limitations of the small sample size, our pilot study showed no significant differences between the two treatment groups *Serenoa repens* extract Me180 and tamsulosin, in 19 patients over 26 weeks. Within the treatment group, there was a moderate improvement of symptoms and Q_{max} in particular up to week 8 of the study. Thus, the clinical significance of these small degrees of statistically significant improvements remains uncertain. Confirming previous studies investigating several SRE's, the investigated extract was well tolerated and investigated laboratory values remained unchanged, leading us to assume that the treatment was safe.

Our study lacks sufficient numbers of participants for assessment of efficacy. In favour of the present results are the rigid inclusion and exclusion criteria, clearly defining the patient population. The study used blinding, an active control and randomisation, had a high compliance and a low withdrawal rate. The dosage of both regimens was according to the recommendations of the suppliers. In the present pilot study, the protocol was evaluated and several of its limitations were found and suggestions for improvement stated. On the basis of the present data, the first study available for Me180, an enlarged study with a larger sample size and longer duration can be designed. Only by such a follow-up study it can be evidenced if the *Serenoa repens* extract Me180 is a reasonable alternative medical therapy for men with obstructive BPH.

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Posters

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