(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 March 2005 (31.03.2005)

(51) International Patent Classification:
A61K 31/593,
A61P 13/08, 13/10

(21) International Application Number:
PCT/EP2004/010760

(22) International Filing Date:

(25) Filing Language:
English

(26) Publication Language:
English

(30) Priority Data:
0322395.5 24 September 2003 (24.09.2003) GB
0325598.1 3 November 2003 (03.11.2003) GB
0416876.1 29 July 2004 (29.07.2004) GB

(71) Applicant (for all designated States except US): BIOCCELL SPA [IT/IT]; via Olgettina 58, I-20132 Milano (IT).

(72) Inventors:
ADORINI, Luciano [IT/IT]; c/o BioCell Spa, via Olgettina 58, I-20132 Milan (IT).
COLLI, Enrico [IT/IT]; c/o BioCell Spa, Via Olgettina 58, I-20132 Milan (IT).

(74) Agent: TEUTEN, Andrew, J.; Sagittarius Intellectual Property Consultants Ltd, Hayes loft, 68A Hayes Place, Marlow, Buckinghamshire SL7 2BT (GB).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KR, KZ, LC, LI, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIP (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— of inventorship (Rule 4.17(v)) for US only
— as to non-prejudicial disclosures or exceptions to lack of novelty (Rule 4.17(v)) for all designations

Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
— with a declaration as to non-prejudicial disclosures or exceptions to lack of novelty

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF VITAMIN D3 ANALOGUE FOR THE TREATMENT OF BENIGN PROSTATIC HYPERPLASIA

(57) Abstract: The use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, for the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia (BPH) and associated symptoms.
USE OF VITAMIN D3 ANALOGUE FOR THE TREATMENT OF BENIGN PROSTATIC HYPERPLASIA

The present invention is concerned with the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholec calciferol (Compound A) for the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia (BPH) and associated symptoms. It is further concerned with a method for preventing and/or treating benign prostatic hyperplasia and associated symptoms by administering 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol in an amount effective to prevent and/or to treat such disease alone or in combination with further active agents.

BPH is a common disorder in elderly men, occurring in approximately 50% of men aged 60 years and in 90% of those aged 85 years. BPH is a specific histopathological entity characterized by stromal and epithelial cell hyperplasia.

For over a century, the two known etiologic factors for the pathogenesis of BPH have been aging and the presence of functional testes. However, as the science of prostate biology advances, this concept becomes inadequate as it does not cover all aspects of BPH pathogenesis. Additional etiologic factors play a significant role in regulating prostatic growth. In particular, evidence has emerged that prostatic growth is under the immediate control of specific growth factors produced by prostatic cells, acting locally on adjacent cells in a paracrine mechanism or to the same cells in an autocrine mechanism. Therefore much effort is currently being put into identifying therapeutic strategies aimed at inhibiting intraprostatic growth factors.

BPH is a common cause of chronic lower urinary tract symptoms which may affect both the filling (irritative symptoms) and voiding (obstructive symptoms) phases of the micturition cycle. These symptoms affect the social, psychological, domestic, occupational, physical and sexual lives of the patients leading to a profound, negative impact on their quality of life. In addition to this, BPH can cause more acute urological complications, particularly acute urinary retention (AUR), often considered the most serious complication of BPH and less frequently recurrent urinary tract infections, upper urinary tract dilatation, bladder stone formation and recurrent hematuria.

BPH management is associated with extremely high social costs, estimated to be 4 billion dollars in 1993 and projected to be 26 billion dollars in 2003 in the USA alone.

The current medical treatment for BPH has consists of orally administered 5 alpha reductase inhibitors (finasteride and dutasteride, recently approved by the FDA) and alpha 1 receptor antagonists (terazosin, doxazosin, tamsulosin as well as silodosin, A10-8507L, RBx-2258 etc). Each of these therapeutic options is associated with both advantages and disadvantages relating to their different mechanisms of action. While alpha 1 receptor antagonists are very effective in reducing symptoms related to lower urinary tract symptoms (LUTS), they are ineffective in reducing the prostate volume and therefore in preventing BPH-related surgery. Conversely, 5 alpha reductase inhibitors
like finasteride and dutasteride, by decreasing dihydrotestosterone (DHT) formation, reduce prostate size and the need for surgery. In addition, recent results from the seven-year Prostate Cancer Prevention Trial, involving more than 18,000 healthy aged men, demonstrated that finasteride can prevent or delay the appearance of prostate cancer (see Thompson, I.M. et al. New England Journal of Medicine (2003) 349 p 215-224). However, as expected (see Kassabian, V.S. Lancet (2003) 361 p 60-62), finasteride was not free from anti-androgenic adverse effects on sexual function, such as decreased sexual potency, sexual desire and gynecomastia, that substantially lessen its attractiveness as a cancer-preventing agent. In addition, finasteride treatment was associated with an increased detection of high-grade prostate cancer, probably because the finasteride-induced low androgen state selected the most aggressive, androgen-insensitive malignant growing cells (see Scardino, P.T. New England Journal of Medicine (2003) 349 p 297-299).

Thus there is an unmet need for a new class of drugs for medical therapy of BPH, which should be able to prevent acute urinary retention, together with its related need for surgery, by decreasing androgen-induced prostate growth but without directly interfering with androgen receptors (AR), and therefore without anti-androgenic prostatic and extra-prostatic adverse effects, for example, sexual side effects. Such medicaments, by disrupting intra-prostatic growth factor signalling, might be useful not only for treating BPH but also for preventing prostate cancer, possibly without selecting AR-insensitive, malignant clones.

As described herein, the Inventors have determined that the non-hypercalcemic, well-tolerated, vitamin D₃ analogue 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (Compound A), is a primary example of such a medicament, as it can act against BPH in an androgen-receptor independent manner by targeting multiple pathways controlling BPH cell growth, including growth factor-mediated prostate proliferation.

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the activated form of vitamin D₃, is a secosteroid hormone that not only plays a central role in bone and calcium metabolism, but is also involved in the regulation of the immune response and the differentiation and apoptosis of many cell types, including malignant cells.

However, a problem with the therapeutic use of calcitriol is its natural ability to induce hypercalcemia and hyperphosphatemia. Hence, analogues of calcitriol retaining biological activity but devoid of hypercalcemic side effects, have been developed. US Patent 5,939,408 and EP808833 disclose a number of 1,25(OH)₂D₃ analogues including the compound 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (Compound A). US Patent 5,939,408 and EP808833 disclose that the compounds induce differentiation and inhibition of proliferation in various skin and cancer cell lines and are useful for the treatment of hyperproliferative
skin diseases such as psoriasis, neoplastic diseases such as leukemia, breast cancer and sebaceous gland diseases such as acne and seborrheic dermatitis and osteoporosis.

It has now surprisingly been found in several studies conducted by the Inventors that, unlike certain other 1,25(OH)$_2$D$_3$ analogues, the 1,25(OH)$_2$D$_3$ analogue Compound A:

![Compound A](image)

significantly reduces the growth of human BPH cells \textit{in vitro} via induction of their apoptosis and reduces prostatic growth \textit{in vivo}, with no effects on testosterone and dihydrotestosterone levels. Furthermore, this inhibition of prostate growth is achieved at non-hypercalcemic doses. Thus, Compound A is an effective pharmacologic agent for the treatment of benign prostatic hyperplasia.

As described in the Examples herein, Compound A reduces prostate size. Furthermore, as observed with finasteride, Compound A counteracts the \textit{in vitro} and \textit{in vivo} proliferative activity of testosterone. Significantly however, and unlike finasteride, Compound A does not inhibit type-1 or type-2 5 alpha-reductase activity and can counteract not only testosterone but even dihydrotestosterone induced BPH cell growth. These anti-androgenic properties of Compound A are independent from interaction with the AR, as shown by the failure of Compound A both to bind to the AR, or to act as an AR agonist or antagonist. Furthermore, Compound A does not affect sex hormone secretion. Furthermore, in our studies, Compound A has no significant effect on prostate specific antigen (PSA) levels thus there appears to be no danger of treatment with Compound A masking this important indicator of possible prostate cancer.

A further significant advantage of Compound A is that \textit{in vitro} studies have revealed that this drug, unlike finasteride, is capable of inhibiting the basal and testosterone-stimulated growth of bladder cells and is expected to be useful in preventing and/or treating of bladder dysfunction in humans. \textit{In vivo} studies in a validated rat bladder outlet obstruction model of bladder dysfunction have also
demonstrated the beneficial effect of Compound A. This is significant because bladder dysfunction is a common and troublesome sequela of BPH. Thus Compound A is capable of reducing prostate size and ameliorating bladder dysfunction i.e. improving bladder function and bladder related symptoms of BPH at the same time through direct effect of Compound A both on the prostate and on the bladder. This effect is expected to go beyond the improvement in bladder symptoms that would be expected merely as a result of prostate size reduction. Bladder symptoms include overactive bladder and indicators of improved bladder function include reduction in non-voiding contractions and residual urine.

Thus, the present invention provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol in the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia. Also considered within the scope of the invention are pharmaceutically acceptable esters and salts of compound A.

The invention thus provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, in the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia.

The invention also provides a method for preventing and/or treating benign prostatic hyperplasia, in patients in need of such prevention or treatment comprising administering a therapeutically effective amount of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof thereby to effect prevention and/or treatment of benign prostatic hyperplasia.

The invention also provides such a method which further comprises the step of obtaining or synthesising the 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof. Typically the compound is formulated together with a pharmaceutically acceptable diluent or carrier.

The invention also provides a kit containing 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof together with instructions directing administration of said compound to a patient in need of prevention and/or treatment of benign prostatic hyperplasia thereby to prevent and/or treat benign prostatic hyperplasia in said patient.

The invention also provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, for the prevention and/or treatment of benign prostatic hyperplasia.
The invention also provides 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-
bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, for use in the prevention and/or treatment of benign prostatic hyperplasia.

The invention also provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-
26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, for the prevention and/or treatment of benign prostatic hyperplasia without anti-androgenic prostatic and extra-prostatic adverse effects. It also provides a method for preventing and/or treating benign prostatic hyperplasia without anti-androgenic prostatic and extra-prostatic adverse effects, in patients in need of such prevention or treatment, comprising administering a therapeutically effective amount of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof.

The invention also provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-
26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, for the prevention and/or treatment of benign prostatic hyperplasia together with concurrent prevention and/or treatment of bladder dysfunction. It also provides a method for preventing and/or treating benign prostatic hyperplasia with concurrent prevention and/or treatment of bladder dysfunction, in patients in need of such prevention or treatment, comprising administering a therapeutically effective amount of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof.

1-Alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol is a known compound and its preparation is described in US Patent 5,939,408, the description of which is incorporated herein by reference.

Esters include pharmaceutically acceptable labile esters that may be hydrolysed in the body to release Compound A.

Salts of Compound A include adducts and complexes that may be formed with alkali and alkaline earth metal ions and metal ion salts such as sodium, potassium and calcium ions and salts thereof such as calcium chloride, calcium malonate and the like.

Compound A, or salt or ester thereof, can be used as a monotherapy or it can be administered in combination with known BPH-active agents, for example an alpha-adrenergic receptor blocking agent such as an alpha 1 receptor antagonist (for example terazosin, doxazosin or tamsulosin or else silodosin, AIO-8507L or RBx-2258) or a 5 alpha-reductase inhibitor (for example finasteride or dutasteride). The expression “BPH-active agent” includes those agents capable of or known to have activity in treating or preventing BPH such as the aforementioned example substances. The combination partner can be admixed with the compound A or its salts or esters in various ratios and can be administered separately, sequentially or simultaneously in separate or combined pharmaceutical formulations. Appropriate doses of known
therapeutic agents will be readily appreciated by those skilled in the art. Combination
of Compound A with two or more (e.g. 3) BPH-active compounds may be envisaged,
e.g. combination with an alpha 1 receptor antagonist and a 5 alpha-reductase inhibitor.
Combination of Compound A with two or more BPH-active compounds is unknown in
the art and represents an aspect of the invention. When administered in combination
with Compound A (or salt or ester) the combination partner(s) may be used at lower
doses compared to that used when the combination partner is administered alone,
perhaps even a dose which is sub-therapeutic when administered alone.

Thus the invention also provides the use as defined above wherein the
medicament is administered separately, sequentially or simultaneously in separate or
combined pharmaceutical formulations with a second BPH-active agent.

Thus the invention also provides the use of 1-alpha-fluoro-25-hydroxy-16,23E-
diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or
erster thereof, in combination with a second BPH-active agent in the manufacture of a
medicament for the prevention and/or treatment of benign prostatic hyperplasia.

The invention also provides a method for preventing and/or treating benign
prostatic hyperplasia, in patients in need of such prevention or treatment comprising
administering a therapeutically effective amount of 1-alpha-fluoro-25-hydroxy-16,23E-
diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or
erster thereof separately, sequentially or simultaneously in separate or combined
pharmaceutical formulations with a second BPH-active agent.

The combinations referred to above may conveniently be presented for use in the
form of a pharmaceutical formulation. The pharmaceutical formulations thus produced
also represent a further aspect of the invention.

We also envisage that the aspect of the invention relating to combination therapy
in BPH may also be extended to other vitamin D compounds (i.e other vitamin D
receptor agonists such as vitamin D, particularly vitamin D3 and analogues thereof).
Thus we also provide a combination therapy comprising use of a vitamin D compound
in combination with one or more other BPH-active compounds e.g. an alpha adrenergic
receptor blocker and/or a 5-alpha reductase inhibitor in the prevention and/or treatment
of BPH. We also provide a pharmaceutical formulation containing a vitamin D
compound in combination with one or more other BPH-active compounds e.g. an alpha
adrenergic receptor blocker and/or a 5-alpha reductase inhibitor optionally together with
a pharmaceutically acceptable diluent or carrier. Examples of alpha adrenergic

receptor blockers and 5-alpha reductase inhibitors are as above mentioned.

Dosage levels and time course of administration of the active ingredients in the
pharmaceutical formulations of the invention may be varied so as to obtain an amount
of the active ingredient which is effective to achieve the desired therapeutic response
for a particular patient, composition, and mode of administration, without being toxic to
the patient. An exemplary dose range of Compound A is from 0.1 to 300 ug per day, for example 50-150 ug per day, e.g. 75 or 150 ug per day. A unit dose formulation preferably contains 50-150 ug, e.g. 75 or 150 ug, and is preferably administered once per day.

Specifically, a preferred dose of Compound A is the maximum that a patient can tolerate and not develop hypercalcemia or other undesirable side effects such as hypercalcuria. Preferably Compound A is administered at a concentration of about 0.001 ug to about 100 ug per kilogram of body weight, about 0.001 – about 10 ug/kg or about 0.001 ug – about 100 ug/kg of body weight. Ranges intermediate to the above-recited values are also intended to be part of the invention.

As noted above, Compound A may be administered as a pharmaceutically acceptable salt or ester thereof however preferably Compound A is employed as is i.e. it is not employed as an ester or a salt thereof.

This dosage may be delivered in a conventional pharmaceutical formulation by a single administration, by multiple applications, or via controlled release, as needed to achieve the most effective results, preferably once or twice daily (especially once daily) e.g. by mouth. In certain situations, alternate day dosing may prove adequate to achieve the desired therapeutic response.

The selection of the exact dose and formulation and the most appropriate delivery regimen will be influenced by, inter alia, the pharmacological properties of the formulation, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient.

Representative delivery regimens include oral, parenteral (including subcutaneous, intramuscular and intravenous), rectal, buccal (including sublingual), pulmonary, transdermal, and intranasal, most preferably oral. Administration may be continuous or intermittent (e.g. by bolus injection).

The invention also provides a pharmaceutical composition comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, and a pharmaceutically acceptable carrier for the prevention and/or treatment of benign prostatic hyperplasia.

The invention also provides a packaged formulation which includes a pharmaceutical composition comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, and a pharmaceutically-acceptable carrier packaged with instructions for use in the treatment of benign prostatic hyperplasia.

As mentioned above, such compositions may be prepared for parenteral (subcutaneous, intramuscular or intravenous) administration, particularly in the form of liquid solutions or suspensions; for oral or buccal administration, particularly in the form
of tablets or capsules; for pulmonary or intranasal administration, particularly in the form of powders, nasal drops or aerosols; and for rectal or transdermal administration.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA., (1985). Formulations for parenteral administration may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered spray. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

Orally administrable compositions may comprise one or more physiologically compatible carriers and/or excipients and may be in solid or liquid form. Tablets and capsules may be prepared with binding agents, for example, syrup, acacia, gelatin, sorbitol, tragacanth, or poly-vinylpyrolidone; fillers, such as lactose, sucrose, corn starch, calcium phosphate, sorbitol, or glycine; lubricants, such as magnesium stearate, talc, polyethylene glycol, or silica; and surfactants, such as sodium lauryl sulfate.

Liquid compositions may contain conventional additives such as suspending agents, for example sorbitol syrup, methyl cellulose, sugar syrup, gelatin, carboxymethylcellulose, or edible fats; emulsifying agents such as lecithin, or acacia; vegetable oils such as almond oil, coconut oil, cod liver oil, or peanut oil; preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Liquid compositions may be encapsulated in, for example, gelatin to provide a unit dosage form.

Preferred solid oral dosage forms include tablets, two-piece hard shell capsules and soft elastic gelatin (SEG) capsules. SEG capsules are of particular interest because they provide distinct advantages over the other two forms (see Seager, H., "Soft gelatin capsules: a solution to many tableting problems"; Pharmaceutical Technology, 9, (1985)). Some of the advantages of using SEG capsules are: a) dose-content uniformity is optimized in SEG capsules because the drug is dissolved or dispersed in a liquid that can be dosed into the capsules accurately; b) drugs formulated as SEG capsules show good bioavailability because the drug is dissolved, solubilized or dispersed in an aqueous-miscible or oily liquid and therefore when released in the body the solutions dissolve or are emulsified to produce drug dispersions of high surface area; and c) degradation of drugs that are sensitive to oxidation during long-term storage is prevented because the dry shell of soft gelatin provides a barrier against the diffusion of oxygen.
The dry shell formulation typically comprises of about 40% to 60% concentration of gelatin, about a 20% to 30% concentration of plasticizer (such as glycerin, sorbitol or propylene glycol) and about a 30 to 40% concentration of water. Other materials such as preservatives, dyes, opacifiers and flavours also may be present. The liquid fill material comprises a solid drug that has been dissolved, solubilized or dispersed (with suspending agents such as beeswax, hydrogenated castor oil or polyethylene glycol 4000) or a liquid drug in vehicles or combinations of vehicles such as mineral oil, vegetable oils, triglycerides, glycols, polyols and surface-active agents.

In an example formulation, the soft gelatin capsules are size 2, white, opaque, oval gelatin capsules containing a liquid fill consisting of the active ingredient, Compound A, dissolved in Miglyol 812 (triglyceride of fractionated C₈-C₁₂ coconut oil fatty acids) with butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA), as preservatives. The soft gelatin capsules can be formulated to contain between 0.01 and 25mg, e.g. 75 or 150 µg of Compound A. Soft gelatin capsules should be stored at 2 – 8°C and protected from light.

Formulations containing Compound A, or a pharmaceutically acceptable salt or ester thereof, optionally in combination with a second BPH-active agent, may be prepared by mixing the ingredients. Alternatively two pharmaceutical formulations may be combined within a single dosage form, for example within the same dual capsule, or by overencapsulation of two different tablets or capsules into a single capsule.

Formulations containing a third or further BPH-active agent may be prepared by an analogous manner.

Formulations are preferably prepared under nitrogen in amber light.

The present invention will now be described with reference to the following non-limiting examples, with reference to the figures, in which:

Figure 1 shows inhibition of BPH cell proliferation by calcitriol and Compound A ("Cmpd A"). Panel A Incubation for 48 h with increasing concentrations (10⁻¹⁸-10⁻⁷ M) of calcitriol (circles) or Compound A (squares) resulted in a significant and dose-dependent inhibition of BPH cell growth (*P<0.01 vs control). ALLFIT analysis indicates that the two secosteroids share the same maximal inhibition (lₘₐₓ=43±1%), but show a marked difference in the rank of potency (-logIC₅₀ Compound A=15.8±0.3; -logIC₅₀ calcitriol=10.2±0.6, P<0.005). Results are expressed as % inhibition (mean±SEM) over their relative controls in 3 different experiments performed in triplicate. Panel B Effect of increasing concentrations (10⁻¹⁸-10⁻⁷ M) of Compound A on BPH cell proliferation stimulated by T (10 nM, squares), KGF (10 ng/ml, circles) or Des(1-3)IGF-I (10 ng/ml, triangles). Compound A induced a significant inhibition (*P<0.01 vs T- or GF-treated cells) of BPH cell growth also in presence of all the stimuli tested with similar lₘₐₓ=66.6±7.3%. However, Compound A was more potent in inhibiting the effect of T (-log IC₅₀=16.4±0.6), than of the other two GFs (-log IC₅₀
Des(1-3)IGF-I = 12.7 ± 0.6, and -log IC_{50} KGF= 14.2 ± 0.6, P<0.0001. Results are expressed as % variation (mean ± SEM) over the maximal stimulation in 3 different experiments performed in triplicate.

**Figure 2** shows the effect of Compound A, cyproterone acetate and finasteride on 5 androgen-stimulated BPH cell growth. BPH cells were incubated for 48 h with Compound A (1 nM) or anti-androgens (finasteride, F, 1 nM; cyproterone acetate, Cyp, 100 nM) in the presence of T (10 nM, Panel A) or DHT (10 nM, Panel B). Results obtained in unstimulated BPH cells are also shown (Panel A). Results are expressed as % variation (mean ± SEM) over their relative controls in three different experiments performed in quadruplicate. Compound A and cyproterone acetate significantly blocked both T- and DHT-induced growth, while finasteride was effective only against T. *P<0.01 vs control; **P<0.01 vs androgen-treated cells.

**Figure 3** shows the lack of agonistic or antagonistic properties of Compound A on human AR. The AR deficient PC3 cell line stably transfected with the human AR was 15 plated in 24 well plates at a density of 2x10^4 cells/well. After 24 h, the cells were transfected with the AR-responsive plasmid pLSPP and, 48 h later, cells were incubated with increasing concentrations of DHT (squares) or Compound A (circles) (Panel A), or with a fixed concentration of DHT (3 nM) in the presence of bicalutamide (squares) or Compound A (circles) (Panel B) for 18 h. Results (the mean of three 20 transfection experiments) are expressed as percentage of bioluminescence per µg of total proteins. To evaluate agonistic activity 100% luciferase activity was set in the presence of DHT 100 nM (Panel A), whereas to test antagonistic activity 100% luciferase activity was set with DHT 3 nM (Panel B).

**Figure 4** shows inhibition of rat ventral prostate growth by Compound A or 25 finasteride. Panel A: Castrated rats, injected with T enanthate (30 mg/Kg/week), were orally treated for 5 day/week for two consecutive weeks with vehicle or with increasing doses of Compound A (10, 30, 100 and 300 µg/Kg) or finasteride (F, 10 and 40 mg/Kg). Ventral prostate weight is expressed as % variation (mean ± SEM) of the weight of intact, vehicle-treated, rats (**P<0.05, *P<0.01 vs control rats, **P<0.01 vs T-supplemented rats). Panel B: Intact adult rats were orally treated for over one month (5 times/week for a total of 27 administration) with vehicle (control) or increasing concentrations of Compound A (10, 30, 100 and 300 µg/Kg) or finasteride (F, 10 and 40 mg/Kg). Ventral prostate weight is expressed as % variation (mean ± SEM) of the weight of control, vehicle-treated rats (**P<0.01 vs control rats).

**Figure 5** shows the effect of Compound A and finasteride on clusterin gene expression in the rat ventral prostate. Panel A: Northern analysis of clusterin mRNA expression in the ventral prostate of vehicle-treated intact (lane 1) or orchidectomized (lane 2) rats. Lanes 3-6 show clusterin gene expression in orchidectomized rats supplemented for two weeks with T enanthate (30 mg/Kg) and orally treated with
vehicle (lane 3), Compound A (300 ug/Kg, lane 4 and 100 ug/Kg, lane 5) or finasteride (40 mg/Kg, lane 6). Every lane was loaded with 10 ug of total RNA. The corresponding GAPDH expression and the ethidium bromide staining of the gel are shown below the blot. The blot is representative of two separate experiments. Panel B: Northern analysis of clusterin mRNA expression in the ventral prostate of adult intact rats orally treated for over 1 month (5 times/week, 27 administrations) with vehicle (lane 1), increasing concentrations of Compound A (10, 30, ug/Kg, lane 2 and 3) or finasteride (40 mg/Kg, lane 4). Every lane was loaded with 10 ug of total RNA. The corresponding GAPDH expression and the ethidium bromide staining of the gel are shown below the blot. The blot is representative of two separate experiments.

Figure 6 shows the morphological effects of Compound A on ventral prostate of castrated and T-supplemented rats. Panels A, B, C and E: Representative fields obtained from cross-sections of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with haematoxylin. In vehicle-treated rats castrated 4-days earlier, clusterin labelling is detectable in the cytoplasm of the atrophic cuboidal epithelial cells (Panel A, 10x). After two-week T supplementation (Panel B, 10x), almost all the clusterin labelling disappeared. Conversely, clusterin positive cells were still present in rats treated with T and different doses of Compound A (100 ug/Kg, Panel C; 300 ug/Kg, Panel E, black arrows). Panels D and F shows sections consecutive to those in Panel C and E, to highlight DNA fragmentation as assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL). Two-week treatment (9 administrations) with Compound A (100 ug/Kg, Panel D; 300 ug/Kg, Panel F) induced massive apoptosis in the majority of epithelial and stromal cells. Note (black arrows), that all the clusterin-positive cells were undergoing apoptosis, while also a consistent portion of clusterin unlabeled cells shows nuclear fragmentation.

Figure 7 shows the morphological effects of Compound A and finasteride on the prostate of intact, adult rats. Panels A, B, D, E, F and H: Representative fields obtained from cross-sections of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with haematoxylin. In Panel A (10x) the primary antibody was omitted. Panel B (10x) shows that in untreated adult rats only few, scanty epithelial cells were labelled in some glands (black arrows). Conversely, in prostate glands from rats treated with increasing concentrations of Compound A cuboidal epithelial cells showing the hallmark of atrophy were dose-dependently stained for clusterin (see black arrows, 10 ug/Kg, Panel D; 30 ug/Kg, Panel E, 100 ug/Kg, Panel F, 10x). Similar results were obtained with finasteride (40 mg/Kg, Panel H, 10x). Panels C, G and I shows serial, consecutive slices to those depicted in Panel B, D and F, respectively, to highlight DNA fragmentation as assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL). Note
(black arrows), that all the clusterin-positive cells were undergoing apoptosis, while also a consistent portion of clusterin unlabeled cells shows nuclear fragmentation.

Figure 8 shows results of a chronic toxicity study in dogs. A clear reduction of prostate weight is shown after 9 months of treatment with Compound A relative to placebo.

Figure 9 shows results of a chronic toxicity study in dogs. A reduction of prostate weight after recovery from treatment with Compound A relative to placebo.

Figure 10 shows the effect of Compound A on testosterone-stimulated bladder cell growth. "hB" = human bladder

Figure 11 shows the effect of Compound A and other comparator compounds on stimulated and basal bladder cell growth. "T 10 nM" = testosterone; F 1nM" = finasteride.

Figure 12 shows the effect of Compound A on bladder weight.

Figure 13 shows the effect of Compound A on spontaneous non-voiding contraction frequency.

Figure 14 shows the effect of Compound A on spontaneous non-voiding contraction amplitude.

Figure 15 shows the effect of Compound A on micturition pressure.

Figure 16 shows the effect of Compound A on residual urine.

Figure 17 shows the effect of Compound A on the contractile response of bladder strips to EFS (Electrical Field Stimulation).

Figure 18 shows the effect of Compound A when in combination with finasteride on unstimulated BPH cell growth.

EXAMPLES

25 Example 1: Effects of Compound A on BPH cells in vitro

MATERIALS AND METHODS

Materials

Minimum Essential Medium (MEM), DMEM-F12 1:1 mixture, Ham's F12 medium, phosphate buffered saline (PBS), bovine serum albumin (BSA) fraction V, glutamine, geneticine, collagenase type IV, vitamin D₃, testosterone (T), dihydrotestosterone (DHT), cyproterone acetate, β-nicotinamide adenine dinucleotide 3'-phosphate reduced form (NADPH), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and a kit for measuring calcemia were purchased from Sigma (St. Louis, MO). The protein measurement kit was from Bio-Rad Laboratories, Inc. (Hercules, CA). Fetal bovine serum (FBS) was purchased from Unipath (Bedford, UK). Monoclonal anti-rat clusterin antibody (mouse monoclonal IgG) specific for beta-chain was from UPSTATE Biotechnology (Lake Placid, NY). Apop Tag kit for in situ end labelling (ISEL) was from Oncor (MD, USA). CHO 1827 and CHO 1829 were provided by Serono International (Geneva, Switzerland). Instagel plus was purchased from Packard (St Louis, MO).
Finasteride (pure substance) (17β-(N,t-butyl)carbamoyl-4-aza-5α-androst-1-en-3-one) was a kind gift from Merck Sharp & Dohme Reasearch Laboratories (Rahway, NJ).

Bicalutamide was a kind gift from AstraZeneca (AstraZeneca, Milan, Italy). Analogue 1-α-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (Compound A) was provided by Bioxell (Bioxell, Milan, Italy). Keratinocyte growth factor (KGF) was from Pepro Tech EC (London, England) and insulin-like growth factor-I, human, [Des(1-3)]IGF-I was purchased from GroPep Limited (Adelaide, Australia). In Situ Cell Death Detection Kit POD for terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labelling (TUNEL) were from Roche Diagnostics Corporation (Indianapolis, IN).

Plastic ware for cell cultures was purchased from Falcon (Oxnard, CA). Disposable filtration units for growth media preparation were purchased from PBI International (Milan, Italy). Lipofectamine 2000 and Opti-MEM I Medium for luciferase transfection were from Invitrogen, Life Technologies (San Giuliano Milanese, Milan, Italy). Thin-layer chromatography (TLC) silica plates were obtained from Merck (Darmstadt, Germany). Testosterone enanthate (T enanthate) was from Geymonat (Anagni, Italy). Coat-A-Count® Total Testosterone detection kit was purchased from Medical System (Genova Struppa, Italy). Rat luteinizing hormone (rLH) [125I] assay systems were from Amersham Pharmacia Biotech (Piscataway, NJ).

Human BPH cells, prepared, maintained and used as previously described in Crescioli, C. et al. Journal of Clinical and Endocrinology Metabolism (2000) 85 p 2576-2583, were obtained from prostate tissues derived from 5 patients, who underwent suprapubic adenomectomy for BPH, after informed consent and approval by the Local Ethical Committee. Patients did not receive any pharmacological treatment in the 3 months preceding surgery.

5α reductase-transfected CHO-1827 and CHO-1829 cell lines

CHO-1827 and CHO-1829 cells, transfected with 5α reductase type 1 (5αR-1) or type 2 (5αR-2), respectively (see Steers W. Urology (2001) 58 p 17-24), were maintained in Ham's F12 medium supplemented with 5% FCS.

AR-transfected PC3 cell line

Human prostate adenocarcinoma PC3 cells, stably transfected with the plasmid p5HbAR-A containing human androgen receptor (hAR) as previously described (see Bonaccorsi, L. et al. Endocrinology (2000) 141 p 3172-3182), were grown in 75 cm² culture flasks in Ham's F-12 medium containing 50 μg/ml geneticine, 10% FCS, penicillin (100 U/ml) and streptomycin (100 mg/ml).

BPH tissue

Prostatic tissues for binding assay were obtained from patients who underwent suprapubic adenomectomy for BPH. No pharmacological treatment was performed in
the 3 months preceding surgery. After surgery, the tissues were immediately placed in liquid nitrogen and stored at -80°C until processing.

Rat tissues

Rat ventral prostate glands were rapidly excised out, weighed and quickly frozen in dry ice. Immunohistochemistry experiments were performed in 14 μm-thick contiguous cryostatic sections for direct comparison of tissue morphology, clusterin expression and apoptosis localization by TUNEL. For total RNA extraction and Western blot analysis, rat ventral prostates from 4 to 6 animals were pooled.

BPH cell proliferation assay

For all cell proliferation assays, 4x10^4 BPH cells were seeded onto 12-well plates in their growth medium, starved in red- and serum-free medium containing 0.1% BSA for 24 h, and then treated with specific stimuli for 48 h. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as controls. Thereafter, cells were trypsinized, and each experimental point was derived from hemocytometer counting, averaging at least six different fields for each well, as previously reported (see Crescioli, C. et al. Journal of Clinical and Endocrinology Metabolism (2000) 85 p 2576-2583). Experiments were performed using increasing concentrations (10^{-18}-10^{-7} M) of calcitriol or Compound A with or without a fixed concentration of T (10 nM), KGF or Des(1-3)IGF-I (10 ng/ml). Growth assays were also carried out using a fixed concentration of androgens (10 nM) with or without Compound A (1 nM, 10 nM) or the anti-androgens finasteride (F, 1 nM) and cyproterone acetate (Cyp, 100 nM). Growth assays were also performed using a fixed concentration of T (10 nM) or GFs (10 ng/ml) with or without Compound A (10 nM). In the same experiment, each experimental point was repeated in triplicate or quadruplicate and experiments were performed 3 times.

Results are expressed as % variation (mean±SEM) over the maximal T or GF-induced stimulation.

In situ end labeling (ISEL)

ISEL was performed onto BPH cells using Apop Tag in situ apoptosis detection kit peroxidase following the manufacturer’s instruction. Cells were incubated with T (10 nM), KGF (10 ng/ml) or Des(1-3)IGF-I (10 ng/ml) with or without Compound A (10 nM). The percentage of apoptotic cells (the number of stained cells divided by the total number of cells) was calculated in at least five separate fields per slide in five different slides. Results are expressed as mean±SEM from three separate experiments.

5α reductase inhibition test

5α reductase inhibition assay was performed using CHO 1827 cells, transfected with 5αR-1, or CHO 1829 cells, transfected with 5αR-2, as described (see Guarna, A. et al. Journal of Medicinal Chemistry (2000) 43 3718-3735). Compound A was added in a concentration range from 10^{-9} to 10^{-5} M, using finasteride as a control inhibitor in each experiment.
Binding assay

Binding assay on cytosol fractions of BPH fragments were carried out as previously reported (see Crescioli, C. et al. *Endocrinology* (2003) 144 p 3046-3057), (final protein concentration: 1.8 mg/ml). Incubations of cytosolic fractions were carried out with increasing concentration (0.125, 0.25, 0.5, 1 nM) of [³H]-R1881 (specific activity: 83.5 Ci/nmol) in the absence or in the presence ([³H]-R1881:1 nM) of increasing concentrations of cold R1881 (10⁻¹⁰-10⁻⁶ M), DHT (10⁻¹⁰-10⁻⁶ M), T (10⁻¹⁰-10⁻⁶ M), bicalutamide (10⁻¹⁰-10⁻⁴ M), and Compound A (10⁻¹⁰-10⁻⁴ M). To prevent R1881 binding to progesterone receptor, 1 μM triamcinolone acetonide was added to each tube. Separation of bound and unbound ligand was performed as previously described (see Crescioli, C. et al. *Endocrinology* (2003) 144 p 3046-3057). Protein content was determined by the known method of Bradford, using BSA as a standard.

Luciferase assay

PC3 cells stably transfected with human AR were plated in 24-well plates at a 15 density of 2x10⁴ in Ham’s F12 plus 10% FCS. After 24 hours, the cells were transfected with 750 ng/well of pLSPP plasmid containing the wilde-type sequence configuration of the MMTV-LTR linked to the firefly luciferase gene (see Pazzagli, M. et al. *Analytical Biochemistry* (1992) 204 p 315-323.), using Lipofectamine 2000 (1 mg/ml) according to the manufacturer’s instructions. After 48 h, the cells were incubated with DHT (10⁻¹²-20 10⁻⁸ M) or bicalutamide (10⁻⁹-10⁻⁵ M), in the presence of 3 nM of DHT, and with equimolar concentration of Compound A for 18 h. Steroids and Compound A analogue were dissolved in ethanol. Transfected cells incubated with ethanol only served as positive controls.

The Luciferase assay was performed with a Berthold luminometer according to the manufacturer’s instructions (Luciferase Assay System, Promega, Milan, Italy). The cells were lysed directly in the plate with 200 μl of lysis buffer. Luciferase activity was measured on 20 μl of cell lysate for 10 s after addition of 100 μl of luciferine. Total protein measurement was performed on 20 μl of cell lysate. At least three independent assays were done in duplicate.

30 Results

Incubation of BPH cells with increasing concentrations of calcitriol or Compound A inhibits cell growth (Figure 1 panel A). Both compounds inhibited dose-dependently cell proliferation. ALLFIT (see De Lean, A. et al. *American Journal of Physiology* (1978) 235 p E97-E102) analysis indicated that, although maximal inhibition of calcitriol and Compound A (“Cmpd A” in the Figures) was not statistically significantly different (Iₘ₅₀=43±1%), their relative potency was, Compound A being several log units more effective than calcitriol (-logIC₅₀ Compound A=15.8±0.3 vs -logIC₅₀ calcitriol=10.2±0.6, P<0.005).
BPH cell proliferation was significantly increased (P<0.01) by testosterone (T) (156±8%), and growth factors (GF), such as Des(1-3)IGF-I (194±6%) or KGF (183±5%). When cell growth was stimulated for 48 h with T or GFs (Figure 1, Panel B) the inhibitory effect of Compound A was even more pronounced (I_{max}=66.6±7.3%).

Mathematical modelling (see De Lean, A. et al. American Journal of Physiology (1978) 235 p E97-E102) of inhibition curves using the synthetic androgen [³H]-R1881 as labelled ligand. LIGAND analysis (see Munson, P.J. et al. Analytical Biochemistry (1980) 107 p 220-239) of the data indicated that unlabeled R1881, DHT, T, and the AR antagonist bicalutamide completely displaced [³H]-R1881 binding (Table I). Conversely, Compound A did not compete for [³H]-R1881 binding at any concentration tested (Table I). These results were confirmed and extended using a luciferase reporter gene assay. In PC3 cells expressing the full length AR coupled to a luciferase report gene, DHT stimulated a dose-dependent increase in luciferase activity (EC_{50}=2±1.3 nM, Panel A), while bicalutamide inhibited DHT-stimulated activity (IC_{50}=194±80 nM, Panel B). In this system, increasing concentration of Compound A neither stimulated nor inhibited AR-mediated luciferase activity increase (Figure 3). Finally to verify whether or not Compound A interacts with the formation of DHT, the active metabolite of T, we performed experiments in CHO cells transfected with type 1 and type 2 5α reductase. Results were compared to those obtained with finasteride (F). While F inhibited T conversion into DHT with the expected IC_{50}s, (IC_{50} for 5α reductase type 1 =659±100 nM and IC_{50} for 5α reductase type 2, =53.7±11 nM, n=3), Compound A did not interfere with either isoenzyme up to the micromolar range (data not shown).
<table>
<thead>
<tr>
<th>AR Ligand</th>
<th>Affinity constants (K_d nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1881</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>DHT</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>T</td>
<td>1.89 ± 0.94</td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>159 ± 82</td>
</tr>
<tr>
<td>Compound A</td>
<td>&gt; 100000</td>
</tr>
</tbody>
</table>

**Table I** Affinity constants of androgen agonists (R1881, DHT, T), antagonist (bicalutamide) and Compound A in human BPH homogenates as detected by \[^3H\]R1881 binding.

The effect of Compound A in BPH cells was, at least in part, due to activation of programmed cell death as detected by ISEL (n=3, Table II). The percentage of apoptotic nuclei dramatically increased (270%) after a 48 h exposure to 10 nM Compound A (P<0.01 vs control). Conversely, treatment with T (10 nM), or GFs (10 ng/ml) significantly (P<0.01) reduced the number of apoptotic BPH cells as compared to untreated cells (Des(1-3)IGF-I= -42%; KGF=-54%; T= -27%). However, even in the presence of GFs or T, Compound A induced a sustained (more than 250%) and significant (P<0.01) increase in the number of ISEL-positive BPH cells.

**Table II** Effect of Compound A (10 nM), GFs (10 ng/ml) or T (10 nM) on DNA fragmentation in BPH cells. Apoptotic index (%) represents the number of stained nuclei, as detected by ISEL, over BPH cells in each of at least 5 separate fields per slide. Results are expressed as mean±SEM in three separate experiments. Compound A is able to induce apoptosis in untreated BPH cells as well as in BPH cells.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Compound A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.55±0.8</td>
<td>68.44±1.26(^a)</td>
</tr>
<tr>
<td>Des(1-3)IGF-I</td>
<td>10.69±0.6(^a)</td>
<td>45.85±0.66(^a, b, c)</td>
</tr>
<tr>
<td>KGF</td>
<td>8.5±0.42(^a)</td>
<td>44.46±0.57(^a, b, c)</td>
</tr>
<tr>
<td>T</td>
<td>13.56±0.72(^a)</td>
<td>49.06±1.87(^a, b, c)</td>
</tr>
</tbody>
</table>
simultaneously incubated with GFs or T (a: \( P<0.01 \) vs control; b: \( P<0.01 \) vs Compound A-treated cells; c: \( P<0.01 \) vs GF- or T-treated cells).

Example 2: Anti-proliferative properties of Compound A in in vivo models of prostate growth

5 Animal protocols

Male Sprague Dawley rats (28 days old) were purchased from Charles River Laboratories (Calco, Lecco, Italy). All animal experimentation described was conducted in accord with accepted standards of animal care. Castration was performed via the scrotal route under ketamine/xylazine anaesthesia. Three days after castration, rats (5-8 animals per group) were treated or not with T enanthate (30 mg/Kg) in two separate weekly sc injections. Rats were orally treated for 5 days the first week, and 4 days the second week with vehicle (miglyol 812), Compound A (10, 30, 100 and 300 \( \mu \)g/Kg) or finasteride (10 and 40 mg/Kg) for a total of 9 administrations, and sacrificed one day later.

Alternatively, intact, adult male Sprague Dawley rats (weight 250 g) were dosed orally with vehicle (miglyol 812), Compound A (10, 30, 100 and 300 \( \mu \)g/Kg) or finasteride (10 and 40 mg/Kg) 5 days/week for 5 consecutive weeks and for two additional days the 6\(^{th}\) week, for a total of 27 administrations, unless otherwise specified. Blood for calcium and hormone measurements was obtained at the end of each experimental protocol.

Northern Hybridisation analysis

Total RNA was extracted using RINFast from Molecular System (San Diego, CA). Blotting, labelling, hybridization conditions and probes (rat clusterin 1.5 Kb full-length cDNA and GAPDH 1.2 Kb full-length cDNA) were performed according to the reported procedures (Bettuzzi et al, Biochemical Journal, (1989), 257, p 293-296 and Marinelli et al, Biochemistry and Cell Biology, (1994), 72, p 515-521). Quantitation of the autoradiograms was obtained by densitometric scanning using an LKB Ultrascan XL densitometer.

Immunohistochemistry

All the cryostatic sections obtained from controls and treated rats were processed in parallel as previously described (Astancolle et al, Journal of Endocrinology, (2000), 167, p 197-204). For every experimental condition, 3 alternate sections from 3 different rat prostates were examined. Negative controls, made by excluding the specific antibody from the reaction, showed no specific staining. Counterstaining was performed with haematoxylin, and cover slips were mounted with Eukitt (O. Kindler GmbH & Co, Germany). Digital high-magnification colour images were acquired by a CCD camera through the microscope.

In situ DNA fragmentation analysis (TUNEL)
DNA fragmentation in prostate cryostatic sections, assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL), was performed using the In Situ Cell Death Detection Kit (POD, Roche) as recommended by the manufacturer. TUNEL positive apoptotic nuclei were documented by digital high-magnification colour images acquired by a CCD camera through the microscope. Counterstaining was performed with eosin, and cover slips were mounted with Eukitt (O Kindler GmbH & Co, Germany).

**Calcium measurements**

Serum calcium levels were measured with a commercially available colorimetric assay (Sigma), according to the manufacturers' instructions.

**Testosterone and rLH measurement**

Serum levels of T and rLH hormones were determined by commercially available radioimmunoassay kits, according to the manufacturers' instructions. To measure serum T in rats, samples were first added to 4 volumes of diethyl ether, mixed by gentle inversion for 15 min and then centrifuged for 5 min at 2000 rpm. The aqueous phase was frozen in dry ice and the organic phase was recovered and evaporated to dryness under a nitrogen stream. The dried extract was reconstituted in the assay buffer as follows: (1 vol: 1 vol) in intact rat, and (4 vol: 1 vol) in castrated rats.

**Statistical analysis**

Statistical analysis was performed by one-way ANOVA and paired or unpaired Student's t tests, when appropriate. Binding data were analysed using the computerized program LIGAND (Munson et al, *Analytical Biochemistry*, (1980), **107**, p 220-139).

The computer program ALLFIT (DeLean et al, *American Journal of Physiology*, 1978, **235**, p E97-E102) was used for the analysis of sigmoid dose-response curves to obtain estimates of half-maximal inhibition values (IC$_{50}$) and half-maximal stimulatory values (EC$_{50}$) as well as maximal inhibitory (I$_{max}$) and stimulatory (E$_{max}$) effects. Data were expressed as (mean±SEM).

**Results**

To test the anti-proliferative properties of Compound A in *in vivo* models of prostate growth, castrated and intact rats were orally treated with increasing concentrations of Compound A (10-300 µg/Kg) or finasteride (F) (10, 40 mg/Kg). As shown in Figure 4, Panel A, castration dramatically reduced ventral prostate weight, while a two-week treatment with testosterone (T) enanthate (30 mg/Kg) not only completely restored, but further stimulated its growth. Compound A, at any dose tested, completely blunted T-stimulated prostate over-growth, reducing ventral prostate weight below that of untreated rats. Similar results were obtained with finasteride (10, 40 mg/Kg). A one-month treatment of intact adult rats with Compound A significantly decreased ventral prostate weight, with a maximal reduction (30%) at the highest dose
tested (300 µg/Kg). At this dose, the inhibitory effect of Compound A on prostate
growth was comparable to that induced by 10 or 40 mg/Kg finasteride (Figure 4, Panel
B). In all the experimental protocols, oral administration of different doses of
Compound A caused a very modest hypercalcemia only at the highest dose tested (300
5 µg/Kg) (Table III). No other discernible side effects were observed.

<table>
<thead>
<tr>
<th></th>
<th>calcemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.2 ± 0.16</td>
</tr>
<tr>
<td>Compound A 10 µg/Kg</td>
<td>10.16 ± 0.24</td>
</tr>
<tr>
<td>Compound A 30 µg/Kg</td>
<td>9.87 ± 0.15</td>
</tr>
<tr>
<td>Compound A 100 µg/Kg</td>
<td>10.55 ± 0.18</td>
</tr>
<tr>
<td>Compound A 300 µg/Kg</td>
<td>10.85 ± 0.1</td>
</tr>
</tbody>
</table>

*Table III* Calcemia (mg/dl) in T-replaced castrated rats after different doses (10, 30, 100, 300 µg/Kg) of Compound A. Compound A never changed calcium serum levels in castrated rats replaced with T enanthate (30 mg/Kg/week) as compared to controls.

Similar results were obtained in intact rats (not shown). Results represent the mean±SEM of rats/group.

To better understand the molecular mechanisms underlying Compound A-induced prostate weight reduction, the expression of clusterin gene and protein and the morphological hallmarks of apoptosis were evaluated by terminal deoxynucleotidyl
transferase (TdT) mediated dUTP nick end-labelling (TUNEL). Clusterin is an
ubiquitous product gene, strictly related to cell cycle arrest and atrophy, the expression
of which is down regulated by androgens. Figure 5 Panel A, shows the prostatic
expression of clusterin mRNA, as detected by Northern analysis, in orchidectomized
rats supplemented or not supplemented with T. Castration dramatically up-regulated
clusterin mRNA abundance, while this effect was completely reverted by a two-week
administration of T. The simultaneous treatment with different concentrations of
Compound A (300 and 100 µg/Kg) or F (40 mg/Kg) partially blunted the T-induced
down-regulation of clusterin gene expression. In intact rats (Figure 5 Panel B), a one-
month administration of different concentrations of Compound A (30 and 100 µg/Kg)
induced a sustained increase in clusterin gene expression in the prostate, comparable,
or even higher, than that induced by 40 mg/Kg F.

The local expression of clusterin in the prostate of orchidectomized rats is shown
in Figure 6. Castration induced a marked and widespread atrophy in the prostate
gland, and nearly all the cuboidal epithelial cells facing the gland lumen were clusterin
positive (Panel A). T-replacement (Panel B) reverted the morphological hallmarks of
atrophy and consistently reduced clusterin staining. Such an effect was prevented by
the simultaneous administration of Compound A (Panels C and E). Panels D and F
show TUNEL results in sections adjacent to those shown in Panels C and E.
Compound A treatment (100 μg/Kg, panel D and 300 μg/Kg, Panel F) induced an evident nuclear fragmentation in epithelial and stromal cells, and apoptosis was detectable in both clusterin positive and negative cells. The first two panels of Figure 7 show the morphology of the prostate gland of an intact rats processed for clusterin detection, with (Panel A) or without (Panel B) the omission of the primary antibody. Note that clusterin labelling is almost absent in the prostate of untreated adult rats (Panel B), as it is nuclear fragmentation (TUNEL, Panel C). Conversely, treatment with different doses of Compound A induced clusterin expression (Panels D-F) and apoptosis (C, G and I). Panel F shows, for comparison, the effect of finasteride (40 mg/Kg) on clusterin positivity in the prostate gland.

To rule out the possibility that Compound A reduced in vivo prostate growth by interfering with pituitary or testis function, rat luteinizing hormone (rLH) and T serum levels were measured in castrated and intact rats. As expected (Table IV, Panel A), castration significantly reduced T while it increased rLH serum levels. T enanthate (30 mg/Kg) administration (two weeks) completely reverted the effect of orchidectomy. Oral treatment with Compound A (100 and 300 μg/Kg) of T-replaced castrated rats did not significantly affect rLH or T serum levels. Similar results were obtained in intact rats (Table IV, panel B). In fact, chronic administration (1 month) of Compound A (10, 30, 100 μg/Kg) or F (40 mg/Kg) to intact rats did not modify rLH and T serum levels.

<table>
<thead>
<tr>
<th>Panel A</th>
<th>rLH</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (intact rats)</td>
<td>2.36 ± 0.46</td>
<td>11.5 ± 2.44</td>
</tr>
<tr>
<td>Castrated</td>
<td>20.64 ± 6*</td>
<td>0.9 ± 0.32*</td>
</tr>
<tr>
<td>castrated+T-replaced</td>
<td>2.08 ± 0.36</td>
<td>21.25 ± 4.12</td>
</tr>
<tr>
<td>castrated+T-replaced +Compound A 100 μg/Kg</td>
<td>1.8 ± 0.2</td>
<td>11.13 ± 1.02</td>
</tr>
<tr>
<td>castrated+T-replaced +Compound A 300 μg/Kg</td>
<td>3.15 ± 0.65</td>
<td>15.73 ± 2.75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel B</th>
<th>rLH</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (intact rats)</td>
<td>2 ± 0.16</td>
<td>11.98 ± 2.87</td>
</tr>
<tr>
<td>Finasteride</td>
<td>2.2 ± 0.4</td>
<td>18.11 ± 3.23</td>
</tr>
<tr>
<td>Compound A 10 μg/Kg</td>
<td>2.22 ± 0.25</td>
<td>19.13 ± 3.83</td>
</tr>
<tr>
<td>Compound A 30 μg/Kg</td>
<td>2.32 ± 0.36</td>
<td>9.39 ± 2</td>
</tr>
<tr>
<td>Compound A 100 μg/Kg</td>
<td>1.96 ± 0.13</td>
<td>11 ± 2.14</td>
</tr>
</tbody>
</table>

**Table IV** rLH (ng/ml) and T (nM) serum levels in T-replaced castrated (Panel A) or intact (Panel B) rats after treatment with different doses of Compound A. Panel A. Castration significantly reduced serum T (*P<0.01 vs control) while it increased serum
rLH (P<0.05 vs control). After treatment with T enanthate (30 mg/Kg/week) rLH and T serum levels were restored. Compound A at all the doses tested did not significantly affect either rLH or T serum levels. Panel B chronic administration (1 month) of F (40 mg/Kg) or Compound A (10, 30 and 100 μg/Kg) did change neither rLH nor T serum levels in intact rats.

This study demonstrates that Compound A reduces prostate size in intact rats to an extent similar to finasteride. In addition, as with finasteride, Compound A abolishes the in vitro and in vivo proliferative activity of testosterone. However, at variance with finasteride, Compound A does not inhibit type-1 or type-2 5α-reductase activity and can counteract not only T but even DHT induced BPH cell growth. These anti-androgenic properties of Compound A are independent from interaction with the AR, as shown by the failure of Compound A to bind to the AR, and to act as AR agonist or antagonist in AR-transfected PC3 cells. Furthermore, Compound A does not affect sex hormone secretion because, in the rat, gonadotropin and T plasma levels were unchanged by daily administration of Compound A for up to one month. Hence, Compound A acts downstream the AR receptor-ligand interaction. Without wishing to be bound by theory this action most probably occurs via the disruption of testosterone-growth factor cross talk.

Very low concentrations of Compound A were able to completely antagonize not only T-stimulated BPH cell proliferation, but also proliferation induced by the two most important intra-prostatic growth factors: IGF-I and KGF. In addition, even in the presence of T or GFs, Compound A induced apoptosis in BPH cells. The Compound A-induced death program was evident also in the prostate of both intact and T-supplemented orchidectomized rats and was characterized by the diffuse appearance of DNA fragmentation with a concomitant increase in clusterin gene and protein expression. Clusterin is a protein tightly regulated in the prostate by androgens (Bettuzzi et al, Biochemical Journal, (1989), 257, p 293-296). Although clusterin function is still not well understood, it is markedly up-regulated in conditions of gland atrophy (Bettuzzi et al, Oncogene, (2002), 21, p 4328-4334 and Bettuzzi et al, Journal of Endocrinology, (1992), 132, p 361-367) and apoptosis (Leskov et al, Journal of Biological Chemistry, (2003), 278, p 11590-11600). Thus, clusterin induction by Compound A treatment is consistent with the capacity of this compound to inhibit proliferation and induce apoptosis in prostate cells.

In conclusion, this study indicates that Compound A is effective in reducing prostate cell growth in different experimental models.

Example 3: Reduction of prostate weight in healthy dogs treated with Compound A.

A 9-month toxicity study was carried out in four groups of male beagle dogs, which were treated by daily oral gavage with 0.5 μg, 1.5 μg and 5 μg/kg body weight/day of Compound A (in vehicle Miglyol 812) or with vehicle alone. This
treatment was followed by a 2-month recovery period for the group receiving the highest dose, 5 μg, after which prostate weights was measured. In addition to entirely favourable toxicity data, a lower prostate weight was observed at the end of treatment with Compound A (see Figure 8) and after recovery (see Figure 9). The results after 5 recovery were analysed statistically via a one-tailed Student’s t test and were found to be significantly different between Compound A and vehicle (P<0.05). These results further demonstrate the ability of Compound A to reduce prostate size in vivo.

**Example 4: Oral Dosage Form Soft Gelatin Capsule**

A capsule for oral administration is formulated under nitrogen in amber light from 0.01 to 25.0 mg of Compound A in 150 mg of fractionated coconut oil (e.g. Miglyol 812), with 0.015 mg butylated hydroxytoluene (BHT) and 0.015 mg butylated hydroxyanisole (BHA), filled in a soft gelatin capsule.

The capsule is prepared by the following process:

1. BHT and BHA are suspended in fractionated coconut oil (e.g. Miglyol 812) and warmed to around 50 °C with stirring, until dissolved.
2. Compound A is dissolved in the solution from step 1 at 50 °C.
3. The solution from step 2 is cooled to room temperature.
4. The solution from step 3 is filled into soft gelatin capsules.

All manufacturing steps are performed under a nitrogen atmosphere and protected from natural light.

**Example 4A: Oral Dosage Form Soft Gelatin Capsule**

A capsule for oral administration is formulated under nitrogen in amber light: 150μg of Compound A in 150 mg of fractionated coconut oil (Miglyol 812), with 0.015 mg butylated hydroxytoluene (BHT) and 0.015 mg butylated hydroxyanisole (BHA), filled in a soft gelatin capsule.

**Example 4B: Oral Dosage Form Soft Gelatin Capsule**

A capsule for oral administration is formulated under nitrogen in amber light: 75μg of Compound A in 150 mg of fractionated coconut oil (Miglyol 812), with 0.015 mg butylated hydroxytoluene (BHT) and 0.015 mg butylated hydroxyanisole (BHA), filled in a soft gelatin capsule.

**Example 5: Reduction in prostate weight in human clinical trials**

A randomised double blind placebo controlled parallel group study was performed to determine the effect of Compound A (1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol) in patients with BPH.

The principal inclusion criterion was that male patients be diagnosed with BPH and have prostate volume > 40 ml as determined by transrectal ultrasound (TRUS). Statistical methods: primary efficacy analyses were planned on Per-Protocol (PP) Population and, as support, the same analyses were to be done on Intent-to-treat population. Patients evaluable for the Per-Protocol analysis were all randomized
patients compliant to protocol criteria that completed the whole course of study without major protocol violations and have valid assessments of prostate volume. Patients valid for intent-to-treat (ITT) population were all randomized patients who received at least one dose of trial medication and for whom the prostate volume at baseline and at 5 12 weeks visit were available.

All patients randomized who took at least one dose of study drug were evaluated for safety analysis.

The treatment group comparability was assessed at baseline for all patients with descriptive meaning. The data were processed by the Chi-Square test for the categorical variables, and by the ANOVA model for the continuous variables.

Descriptive statistics were calculated by means of usual methods: mean, standard deviation, minimum and maximum values on continuous variables, and absolute and relative frequencies for categorical ones. Descriptive statistics were done per treatment and per visit/weeks.

Centers with less than 4 patients were pooled.

The primary efficacy variable was the percentage change of prostate volume, measured by centralized MRI axial scanning. An ANOVA model was used for the analysis, with treatment and center as fixed effects.

Study participants received 150 µg capsule (as per Example 4A with drug omitted in the case of placebo) once daily in the morning. The treatment period was 12 weeks. The number of patients involved was as follows:

<table>
<thead>
<tr>
<th></th>
<th>Compound A</th>
<th>Placebo</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomised patients</td>
<td>57</td>
<td>62</td>
<td>119</td>
</tr>
<tr>
<td>Complete patients</td>
<td>56 (98.3%)</td>
<td>60 (96.8%)</td>
<td>116 (97.5%)</td>
</tr>
<tr>
<td>Discontinued or lost to follow-up</td>
<td>0</td>
<td>2 (3.2%)</td>
<td>2 (1.7%)</td>
</tr>
<tr>
<td>Unsatisfactory therapeutic effect</td>
<td>1 (1.8%)</td>
<td>0</td>
<td>1 (0.8%)</td>
</tr>
</tbody>
</table>

**Percentage change in prostate volume measured by Axial Scanning**

The percentage change in prostate volume in the PP population was -1.89 ± 5.2 in the Compound A group vs 4.99 ± 5.99 in placebo group with a significant p value of <0.0001 in favour of Compound A. The estimate of difference between treatments (Compound A minus placebo) was -7.24 with 95% confidence limit of -9.54 and -4.94. The centre effect was also significant (p=0.0176). The same analysis performed on the ITT population confirmed the results (p=0001), that is that Compound A was more effective than placebo in reducing the prostate volume.
In patients with a baseline prostate volume \( \geq 80 \) ml the difference between treatment groups (\( p<0.001 \)) was clearer in comparison with patients with a baseline prostate volume <60 ml (\( p=0.0320 \)) especially in the PP population.

In patients with age between 61-70 years the difference between treatments, always in favour of Compound A, was more evident than with respect to older patients (age >70 years). In fact in the ITT population, the difference between treatments in patients with age >70 had a significance of \( p=0.0540 \) vs \( p<0.0001 \) for the other classes of patients.

Responders

The proportion of responders observed with Compound A was 27.5\%, in the PP population, with an unchanged proportion of 65\% and only a 7.5\% of patients not responder. In placebo group the class of responders was null, in fact patients were equitably separated in unchanged and not responder (50\% in each class). The Chi-Square test comparing proportions confirmed the results observed in the primary efficacy variable, \( p<0.0001 \), that is Compound A was more effective than placebo in reduction of prostate volume.

In the ITT population the results were confirmed, the proportion of responders in the Compound A group being 28.8\%, with a chi-square p-value of <0.0001 between treatments.

The mean reduction of prostate volume vs baseline in responder patients was \( -6.88 \pm 2.5 \), in the PP population, while the mean difference in unchanged patients is \( 0.35 \pm 2.3 \) in the Compound A group vs \( 0.40 \pm 2.0 \) in Placebo group. For non responder patients the mean difference was \( 3.93 \pm 0.8 \) in the Compound A group vs \( 7.48 \pm 4.9 \) in Placebo group that confirms the greater efficacy of Compound A in controlling and reducing prostate volume.

Percentage change of Prostate Volume measured by centralized Paraxial and transitional Scanning

The supportive analyses on paraxial and transitional acquisition confirmed the results obtained on axial scanning. In particular, in the PP population, for paraxial acquisition the percentage change was \(-1.30 \pm 6.9\) in the Compound A group vs \(2.57 \pm 6.8\) in placebo group, \( p=0.0172 \); while for transitional scanning the percentage change was \(-0.22 \pm 9.6\) for Compound A group vs \(6.18 \pm 10.9\) for Placebo group, \( p=0.0028 \). Also in the ITT population there was a significant difference between treatments in favour of Compound A, in reduction of prostate volume.

Serum Total PSA and Hormone Levels

The PSA mean change in the Compound A group was \( 0.23 \pm 1.3 \) vs \( 0.43 \pm 1.7 \) in placebo group, for PP population. No significant difference was observed between treatments (\( p=0.2722 \)).
Also for testosterone, in PP population, there was no significant difference between groups (p=0.2150), with mean change 0.07±1.5 in the Compound A group vs 0.22±1.4 in Placebo group.

There was no difference between treatment groups for dihydrotestosterone (p=0.7257 – PP population), with an observed mean change in PP population of -30.77±227.71 in the Compound A group vs -166.76±490.26 in the Placebo group. There was no difference in LH hormone (p=0.9920 – PP population), with a mean change in Compound A of -0.02±1.7 vs -0.00±1.8 in Placebo group for PP population. The mean changes observed in PSA and in hormone levels was around zero, except for DHT, Compound A does not modify the hormone levels.

The same results were confirmed in the ITT population.

**Safety**

The number of patients with at least one adverse event was 31 (17 in the Compound A group, 14 in placebo group); no patient dropped out due to adverse events, and only one patient in Placebo group experienced a serious adverse event: an acute cholecystitis, solved with hospitalization.

The number of patients with adverse events related to Compound A treatment was 3 (5.26%), while the number of patients with adverse events related to treatment was 6 (9.68%) in the Placebo group. The events related to Compound A were: dizziness, headache, libido decrease and hot flushes, while the events related to Placebo were: urine phosphate increase, headache, syncope, libido decrease (3 patients), hypercalciuria, erectile dysfunction and hot flushes.

The calciuria values monitored over the course of the study in the Compound A group did not differ significantly from the Placebo group.

**Conclusion**

In this short proof of concept study for effect of Compound A on prostatic size in patients with BPH, the drug proved to be efficacious. The analysis of the primary variable of the study, namely the evaluation of the prostatic size, showed significant difference between Compound A and placebo, thus confirming that the tested drug is able to arrest the progression of the disease. The safety profile was good, there was no different incidence of adverse events between Compound A and placebo, and no severe adverse event was reported in Compound A group. The tested drug was devoid of any antiandrogenic effects, had no effect on PSA levels, and had no significant effect on the calcium homeostasis.

**Example 6: The activity of Compound A on the growth and function of bladder cells**

Compound A has been shown to be effective in inhibiting the basal and testosterone-stimulated growth of bladder cells. This activity, never reported before, is dose-dependent with a $1.6\pm7\times10^{-15}$ for 1-alpha-fluoro-25-hydroxy-16,23e-diene-26,27-
bishomo-20-epi-cholecalciferol ("Compound A"/"Cmpd A" in the figures) (on stimulated cells) (see Figure 10 and Figure 11).

This effect was significantly greater than that of the anti-androgen finasteride widely used in the treatment of uro-genital diseases (Figure 11).

5 Example 7: The effect of Compound A on bladder dysfunction in a bladder outlet obstruction model

Experimental
1. Materials
1.1. Animals:
10 Female Sprague-Dawley rats, weighing 200-250g
1.2. Grouping:
Group A: BOO rats, treated with Compound A over 2 weeks, beginning at day 1 after creation of the obstruction (n=12)
Group B: BOO rats, treated with vehicle over 2 weeks, beginning at day 1 after creation of the obstruction (n=12)
Group C: Sham operated rats, treated with Compound A over 2 weeks, beginning at day 1 after surgery (n=12)
1.3. Studies:
a) Cystometry (~ 18 hours after last administration of the drug/vehicle, 12 hours after removal of the obstructing ligature) under conscious conditions.
b) Measurements of bladder weight
c) In vitro investigations
2. Methods
2.1. Bladder Outlet Obstruction (BOO):
25 The bladder and urethrovvesical junction were exposed through a lower abdominal midline incision. A 0.9 mm metal rod was placed alongside the proximal urethra and a 3-0 silk ligature was tied tightly around the urethra and the rod, which was consequently removed. Sham surgery was performed accordingly, without placing the ligature. After 13 days the ligature was be removed and a catheter was inserted into the bladder dome and tunneled subcutaneously.
2.2. Cystometry:
The following morning after insertion of the catheter, the cystometric investigation was performed without any anesthesia or restraint in a metabolic cage. The amount of voided urine was measured by means of a fluid collector, connected to a force displacement transducer. The bladder was continuously filled with saline at room temperature. The catheter was also connected to a pressure transducer. After a stabilization period of 30-60 minutes, when reproducible voiding patterns are achieved, the following parameters were recorded over a period of 30 minutes: Basal bladder pressure, micturition pressure, threshold pressure, micturition interval and volume, and
non-voiding contractions. The amount of residual urine was investigated manually 3 times, at the end of the cystometry. Bladder capacity was calculated based on the measured values.

2.3. In vitro investigations:

5 2.3.1 Preparations
After completion of the cystometries, the rats were sacrificed by carbon monoxide asphyxiatiion followed by exsanguination. The abdomen was accessed through a lower midline incision whereafter the symphysis was opened. The bladder was carefully dissected free, and immediately placed in chilled Krebs solution, and strip preparations were dissected.

2.3.2 Recording of mechanical activity
The bladder and urethra were separated at the level of the bladder neck, and semicircular strips were prepared from the middle third of the detrusor (1 x 2 x 5 mm). All preparations were used immediately after removal.

The strips were transferred to 5 ml tissue baths containing Krebs solution. The Krebs solution was maintained at 37°C and bubbled continuously with a mixture of 95% O₂ and 5% CO₂, resulting in a pH of 7.4. The strips were suspended between two L-shaped hooks by means of silk ligatures. One hook was connected to a movable unit allowing adjustment of passive tension, and the other to a Grass FT03C (Grass Instruments Co, MA, USA) force transducer. Isometric tension was recorded using a Grass polygraph (7D). After mounting, the strips were stretched to a passive tension of 4 mN (the same tension for all preparations) and allowed to equilibrate for 45-60 min before further experiments were performed.

2.3.3. Electrical field stimulation
Electrical field stimulation (EFS) was accomplished by means of two platinum electrodes placed on either side of the preparations, and was performed using a Grass S48 or S88 stimulator, delivering single square wave pulses at selected frequencies. The train duration was 5 s, the pulse duration 0.8 ms, and the stimulation interval 2 min. The polarity of the electrodes was shifted after each pulse by means of a polarity changing unit.

2.3.4 Procedure
Each experiment was started by exposing the preparations to a high K⁺ (124 mM) Krebs solution until two reproducible contractions are obtained. Then the following experiments were carried out:

35 a) Electrical stimulation of nerves was performed and frequency-response relations obtained, in the presence and absence of atropine.

b) Concentration-response curves were constructed for carbachol and ATP
The validated bladder outlet obstruction rat model described above was used to test the ability of Compound A to control and treat bladder dysfunction. The objective was to evaluate whether a vitamin D3 analogue (1-alpha-fluoro-25-hydroxy-16,23e-diene-26,27-bishomo-20-epi-cholecalciferol – compound “A”) at the dose of 150 ug/Kg/daily can prevent bladder hypertrophy and bladder dysfunction such as bladder overactivity.

In this model a ligature was surgically placed around the outlet of the catheterized bladder, so that when the catheter was removed, the bladder experienced increased urethral resistance. The rats underwent continuous cystometry to evaluate bladder function. In addition the contractile properties of isolated bladder preparation in response to nerve stimulation and exogenous stimuli in vitro were investigated under electrical field stimulation (EFS).

The following cystometric parameters were investigated (see Figures 12-16): -micturition pressure (the maximum bladder pressure during micturition),
-bladder capacity (residual volume after voiding plus the volume of saline infused to induce the void)
-micturition volume (volume of the expelled urine)
-residual urine (bladder capacity minus micturition volume)

and
-frequency and amplitude of spontaneously occurring changes intravesical pressure (non-voiding contractions).

In this model the analogue under evaluation had a beneficial effect on bladder function. This effect was evident in the normal bladder and is maintained in bladder outlet obstruction. In particular significant differences versus vehicle were observed in:
-spontaneous non-voiding contraction frequency and amplitude (Figures 13 and 14);
-residual urine (absent with Compound A, Figure 16);
-micturition pressure (Figure 15);

In addition a beneficial effect on bladder function has been confirmed in the in vitro tests:
-K response;
-response to EFS (Figure 17);
-response to carbachol.

Finally a slight decrease in bladder weight was observed with Compound A (Figure 12).

These data demonstrate the use of Compound A (in the dose range from 50 ug to 300 ug - equivalent to approximately 0.725 to 5 ug/kg of body mass in humans) in the prevention and treatment of bladder dysfunction, e.g. overactive bladder, such as is, for example, demonstrated in patients with BPH.
Example 8: Effect of Compound A in combination with finasteride on growth of unstimulated BPH cells.

The effect of Compound A (concentration $10^{-13} M - 10^{-17} M$) with and without the 5-alpha reductase inhibitor finasteride (concentration 10 nM) on growth of unstimulated BPH cells was tested. The experimental protocol was essentially as described in Example 1 for the data shown in Figure 2A. The results are shown in Figure 1B. A trend in favour of the drug combination over the individual components can be seen suggesting that the combination therapy may be useful in the treatment of BPH.

Abbreviations

10 T  testosterone
DHT  dihydrotestosterone
GF   growth factor
BPH  benign prostatic hyperplasia
PP   per-protocol
15 ITT intent-to-treat
ANOVA Analysis of Variance
TRUS Transrectal ultrasound
BOO  Bladder Outlet Obstruction
AR   Androgen receptors
20 PSA Prostate Specific Antigen
ug   microgram
ul   microlitre

All references including patent and patent applications referred to in this application are incorporated herein by reference to the fullest extent possible.

Throughout the specification and the claims which follow, unless the context requires otherwise, the word 'comprise', and variations such as 'comprises' and 'comprising', will be understood to imply the inclusion of a stated integer or step or group of integers but not to the exclusion of any other integer or step or group of integers or steps.

The application of which this description and claims forms part may be used as a basis for priority in respect of any subsequent application. The claims of such subsequent application may be directed to any feature or combination of features described herein. They may take the form of product, composition, process, or use claims and may include, by way of example and without limitation, the following claims:
CLAIMS

1. The use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof, in the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia.

2. The use according to claim 1 wherein the medicament is administered separately, sequentially or simultaneously in separate or combined pharmaceutical formulations with a second BPH-active agent.

3. The use of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, in combination with a second BPH-active agent in the manufacture of a medicament for the prevention and/or treatment of benign prostatic hyperplasia.

4. The use according to claim 2 or 3 wherein the second BPH-active agent is an alpha-adrenergic receptor blocking agent.

5. The use according to claim 4 wherein the alpha-adrenergic receptor blocking agent is selected from terazosin, doxazosin, tamsulosin, silodosin, AIO-8507L and RBx-2258.

6. The use according to claim 2 or 3 wherein the second BPH-active agent is a 5 alpha-reductase inhibitor.

7. The use according to claim 6 wherein the 5 alpha-reductase inhibitor is selected from finasteride and dutasteride.

8. A pharmaceutical formulation comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, and a pharmaceutically acceptable carrier for the prevention and/or treatment of benign prostatic hyperplasia.

9. A packaged formulation including a pharmaceutical composition comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, and a pharmaceutically acceptable carrier packaged with instructions for use in the prevention and/or treatment of benign prostatic hyperplasia.

10. The use according to any one of claims 1 to 7 wherein the 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or pharmaceutically acceptable salt or ester thereof, is provided in unit dose form.

11. A formulation according to claims 8 or 9 wherein the 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or pharmaceutically acceptable salt or ester thereof, is provided in unit dose form.

12. A formulation according to claims 8, 9 and 11 further comprising a second BPH-active agent.
13. The use according to claim 10 wherein the unit dose of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol is 50 to 150 ug.
14. The use according to any one of claims 1 to 7 or 10 or 13 for the prevention and/or treatment of benign prostatic hyperplasia without anti-androgenic prostatic and 5 extra-prostatic adverse effects.
15. The use according to any one of claims 1 to 7 or 10, 13 or 14 for the prevention and/or treatment of benign prostatic hyperplasia together with concurrent prevention and/or treatment of bladder dysfunction.
16. A pharmaceutical formulation comprising 1-alpha-fluoro-25-hydroxy-16,23E-10 diene-26,27-bishomo-20-epi-cholecalciferol dissolved in fractionated coconut oil together with one or more preservatives.
17. A formulation according to claim 16 wherein the fractionated coconut oil is Miglyol 812.
18. A formulation according to claim 16 or claim 17 wherein the preservatives are 15 selected from butylated hydroxytoluene, butylated hydroxyanisole and mixtures thereof.
19. A pharmaceutical formulation comprising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof in combination with a second or further BPH-active agent.
20. A formulation according to claim 19 wherein 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester is in combination with an alpha-adrenergic receptor blocking agent.
21. A formulation according to claim 19 wherein 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester is in combination with a 5-alpha reductase inhibitor.
22. A formulation according to claim 21 wherein the 5-alpha reductase inhibitor is finasteride or dutasteride.
23. A formulation according to any of claims 16 to 22 for use in the prevention and/or treatment of benign prostatic hyperplasia.
24. A formulation according to claim 8, 11, 12 or 15 to 23 which is presented as a 30 capsule.
25. 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof, for use in the prevention and/or treatment of benign prostatic hyperplasia.
26. 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof, for use in the prevention and/or treatment of benign prostatic hyperplasia without anti-androgenic prostatic and extra-prostatic adverse effects.
27. 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof, for use in the prevention and/or
treatment of benign prostatic hyperplasia together with concurrent prevention and/or treatment of bladder dysfunction.

28. A formulation according to claim 11 wherein the unit dose of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol is 50 to 150 ug.

29. A method for preventing and/or treating benign prostatic hyperplasia, in patients in need of such prevention or treatment comprising administering a therapeutically effective amount of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof thereby to effect prevention and/or treatment of benign prostatic hyperplasia.

30. A method for preventing and/or treating benign prostatic hyperplasia, in patients in need of such prevention or treatment comprising administering a therapeutically effective amount of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol or a pharmaceutically acceptable salt or ester thereof separately, sequentially or simultaneously in separate or combined pharmaceutical formulations with a second BPH-active agent thereby to effect prevention and/or treatment of benign prostatic hyperplasia.

31. A method according to claim 30 wherein the second BPH-active agent is an alpha-adrenergic receptor blocking agent.

32. A method according to claim 31 wherein the alpha-adrenergic receptor blocking agent is selected from terazosin, doxazosin, tamsulosin, silodosin, AIO-8507L and RBx-2258.

33. A method according to claim 30 wherein the second BPH-active agent is a 5 alpha-reductase inhibitor.

34. A method according to claim 33 wherein the 5 alpha-reductase inhibitor is selected from finasteride and dutasteride.

35. A method according to any one of claims 29 to 34 wherein the 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or pharmaceutically acceptable salt or ester thereof, is provided in unit dose form.

36. A method according to claim 35 wherein the unit dose of 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol is 50 to 150 ug.

37. A method according to any one of claims 29 to 36 for the prevention and/or treatment of benign prostatic hyperplasia without anti-androgenic prostatic and extraprostatic adverse effects.

38. A method according to claim 29 to 37 for the prevention and/or treatment of benign prostatic hyperplasia together with concurrent prevention and/or treatment of bladder dysfunction.

39. A method according to any one of claims 29 to 38 which further comprises the step of obtaining or synthesising 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof.
40. A method according to claim 39 wherein the 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof is formulated in a pharmaceutical composition together with a pharmaceutically acceptable diluent or carrier.

41. A kit containing 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof together with instructions directing administration of said compound to a patient in need of prevention and/or treatment of benign prostatic hyperplasia thereby to prevent and/or treat benign prostatic hyperplasia in said patient.

42. A kit according to claim 41 wherein the 1-alpha-fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol, or a pharmaceutically acceptable salt or ester thereof, is formulated in a pharmaceutical composition together with a pharmaceutically acceptable diluent or carrier.
FIGURES

Figure 1A

- ○ Calcitriol
- □ Cmpd A

Cell number (% of control)

Agonist (M)

$10^{-18}$ $10^{-16}$ $10^{-14}$ $10^{-12}$ $10^{-10}$ $10^{-8}$
Figure 1B

- Des (1-3)IGF-I
- KGF
- Testosterone

Cell number (% of control) vs. Cmpd A (M)

Y-axis: Cell number (% of control)
X-axis: Cmpd A (M)

Logarithmic scale for Cmpd A (M)
Figure 2A

BAR CHART

BPH Cell No. (% of control)

control T T + Cmpd A T + F T + Cyp Cmpd A F Cyp

140 120 100 80 60 40 20 0

0 50 100
Figure 2B

BPH cell number (% of control)

control  DHT  DHT + Cmpd A  DHT + F  DHT + Cyp
Figure 3A

EC₅₀ = 2.02 ± 1.3 nM
n= 4

% of activity

10¹² 10¹¹ 10¹⁰ 10⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶
Figure 3B

\[ IC_{50} = 194 \pm 80 \text{ nM} \]

\[ n = 4 \]

- Cmpd A
- Bicalutamide

% of activity vs. dose of DHT (3 nM)
Figure 4A

Ventral Prostate Weight (% of control)

castrated rats
+T 30 mg/Kg

control

10 30 100 300
Cmpd A (µg/Kg)

1 40
Finasteride (mg/Kg)
Figure 4B

![Bar chart showing ventral prostate weight (% of control) for different treatments.](image)
Figure 5

A
clusterin -
GAPDH -

B
clusterin -
GAPDH -
Figure 8

Prostate weight (grams)

N=4

Vehicle  Compound A 1,5 μg/kg  Compound A 0,5 μg/kg  Compound A 5 μg/kg

Percent reduction  15  6.4  21
Figure 9

Prostate weight (grams)

N=3

Percent reduction 44
Figure 16

![Graph showing microliter comparison between BOO-Vehicle and BOO-Drug.]

*sign. different from BOO-Vehicle

Figure 17

![Graph showing percent of potassium contraction against frequency in Hz.]

*sign. different from BOO-Vehicle
Figure 18

F=finasteride (10 nM)

*P<0.02 vs control
°P<0.01 vs F-treat
^P<0.01 vs Cmpd A-treat (equimolar)

Cell number (% of control)

- control
- F
- F+Cmpd A 1E-17
- F+Cmpd A 1E-15
- F+Cmpd A 1E-13
- Cmpd A 1E-17
- Cmpd A 1E-15
- Cmpd A 1E-13
Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)):

In relation to this international application BioXell SpA declares that the subject matter contained within this application was disclosed as follows:

(i) kind of disclosure:
   (c) abuse

(ii) date of disclosure:
   30 July 2003 (30/07/2003)

(iii) title of disclosure:

(iv) place of disclosure:

(v) this declaration is made for the purposes of:
   (a) all designations

☐ This declaration is continued on the following sheet, “Continuation of Box No. VIII (v)”.

Form PCT/RO/101 (declaration sheet (v)) (January 2004)
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 7 A61K31/593 A61P13/08 A61P13/10

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbol)

IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, CHEMABS Data, SCISEARCH, PHARMAPROJECTS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

**Further documents are listed in the continuation of box C.**

**Patent family members are listed in annex.**

**Date of the actual completion of the international search**

31 January 2005

**Date of mailing of the international search report**

15/02/2005

**Name and mailing address of the ISA**

European Patent Office, P.O. 5618 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3010

Authorized officer

Hornich, E

Form PCT/SA/210 (second sheet) (January 2004)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevan to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P,X</td>
<td>CRESCIOLOI C ET AL: &quot;Inhibition of prostate cell growth by BXL-628, a calcitriol analogue selected for a phase II clinical trial in patients with benign prostate hyperplasia&quot; EUROPEAN JOURNAL OF ENDOCRINOLOGY, vol. 150, no. 4, April 2004 (2004-04), pages 591-603, XP009043025 ISSN: 0804-4643 the whole document</td>
<td>1-42</td>
</tr>
<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim No.</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>A</td>
<td>WO 03/034961 A1 (EURO-CELTIQUE, S.A.; TAVARES, LINO; SHEVCHUK, IHOR; ALFONSO, MARK; MARC) 1 May 2003 (2003-05-01) the whole document</td>
<td>----</td>
</tr>
<tr>
<td>A</td>
<td>WO 03/047595 A1 (NOVACEA, INC.; CHEN, ANDREW-XIAN; FAN, JUN; YU, XI-YUN; WHITEHOUSE, MAR) 12 June 2003 (2003-06-12) paragraph '0036!' - paragraph '0040!' tables 1-3</td>
<td>----</td>
</tr>
</tbody>
</table>
INTERNATIONAL SEARCH REPORT

Box II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. X Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 29-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. □ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found 4 multiple Inventions in this International application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

□ The additional search fees were accompanied by the applicant's protest.

□ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2004)
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AU 723929 B2</td>
<td>07-09-2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 2357797 A</td>
<td>27-11-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BR 9703384 A</td>
<td>15-09-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2205275 A1</td>
<td>23-11-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1175573 A</td>
<td>11-03-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CZ 9701578 A3</td>
<td>17-12-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DE 69730774 D1</td>
<td>28-10-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1464640 A2</td>
<td>06-10-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0808833 A2</td>
<td>26-11-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HR 970273 A1</td>
<td>30-04-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HU 9700926 A2</td>
<td>28-01-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2859247 B2</td>
<td>17-02-1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 10045713 A</td>
<td>17-02-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MA 24251 A1</td>
<td>01-04-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO 972304 A</td>
<td>24-11-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NZ 314839 A</td>
<td>28-01-2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PL 320132 A1</td>
<td>24-11-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SG 70009 A1</td>
<td>25-01-2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TR 9700415 A2</td>
<td>21-12-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TW 445252 B</td>
<td>11-07-2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 5939408 A</td>
<td>17-08-1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 6329538 B1</td>
<td>11-12-2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 6452028 B1</td>
<td>17-09-2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZA 9704336 A</td>
<td>24-11-1997</td>
</tr>
</tbody>
</table>


WO 03034961  A1  01-05-2003  NONE